HPLC analysis of plant sterol oxidation products

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ACADEMIC DISSERTATION

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ABSTRACT

Increased interest in the cholesterol-lowering effect of plant sterols has led to development of plant sterol-enriched foods. When products are enriched, the safety of the added components must be evaluated. In the case of plant sterols, oxidation is the reaction of main concern. *In vitro* studies have indicated that cholesterol oxides may have harmful effects. Due their structural similarity, plant sterol oxidation products may have similar health implications.

This study concentrated on developing high-performance liquid chromatography (HPLC) methods that enable the investigation of formation of both primary and secondary oxidation products and thus can be used for oxidation mechanism studies of plant sterols. The applicability of the methods for following the oxidation reactions of plant sterols was evaluated by using oxidized stigmasterol and sterol mixture as model samples.

An HPLC method with ultraviolet and fluorescence detection (HPLC-UV-FL) was developed. It allowed the specific detection of hydroperoxides with FL detection after post-column reagent addition. The formation of primary and secondary oxidation products and amount of unoxidized sterol could be followed by using UV detection. With the HPLC-UV-FL method, separation between oxides was essential and oxides of only one plant sterol could be quantified in one run. Quantification with UV can lead to inaccuracy of the results since the number of double bonds had effect on the UV absorbance.

In the case of liquid chromatography-mass spectrometry (LC-MS), separation of oxides with different functionalities was important because some oxides of the same sterol have similar molecular weight and moreover epimers have similar fragmentation behaviour. On the other hand, coelution of different plant sterol oxides with the same functional group was acceptable since they differ in molecular weights. Results revealed that all studied plant sterols and cholesterol seem to have similar fragmentation behaviour, with only relative ion abundances being slightly different. The major advantage of MS detection coupled with LC separation is the capability to analyse totally or partly coeluting analytes if these have different molecular weights.

The HPLC-UV-FL and LC-MS methods were demonstrated to be suitable for studying the photo-oxidation and thermo-oxidation reactions of plant sterols. The HPLC-UV-FL method was able to show different formation rates of hydroperoxides during photo-oxidation. The method also confirmed that plant sterols have similar photo-oxidation behaviour to cholesterol. When thermo-oxidation of plant sterols was investigated by HPLC-UV-FL and LC-MS, the results revealed that the formation and decomposition of individual hydroperoxides and secondary oxidation products could be studied. The methods used revealed that all of the plant sterols had similar thermo-oxidation behaviour when compared with each other, and the predominant reactions and oxidation rates were temperature dependent. Overall, these findings showed that with these LC methods the oxidation mechanisms of plant sterols can be examined in detail, including the formation and degradation of individual hydroperoxides and secondary oxidation products, with less sample pretreatment and without derivatization.

PREFACE

This study was carried out at the Department of Applied Chemistry and Microbiology, Food Chemistry Division, at the University of Helsinki. The work was financially supported by The Academy of Finland and The Finnish Graduate School on Applied Bioscience, which is gratefully acknowledged.

My deepest gratitude goes to my supervisors Professor Vieno Piironen and Docent Anna-Maija Lampi. You made this thesis possible, your support throughout my work has been invaluable. Thank you especially for being so patient when helping me with the writing process of this thesis and my publications.

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I also want to thank my friends and relatives. I am deeply grateful to my parents and my sister for all the support and encourangement I have received from them. Finally, I thank my dear husband Tomi for his love and flexibility, and our lovely children Minttu and Ossi for being the sunshine of my live.

Hyvinkää, February 2008

Suvi Kemmo

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by Roman numerals I-IV:

I Säynäjoki, S., Sundberg, S., Soupas, L., Lampi, A.-M. and Piironen, V. 2003. Determination of stigmasterol primary oxidation products by high-performance liquid chromatography. Food Chem. 80:415-421.

II Kemmo, S., Soupas, L., Lampi, A.-M. and Piironen, V. 2005. Formation and decomposition of stigmasterol hydroperoxides and secondary oxidation products during thermo-oxidation. Eur. J. Lipid Sci. Technol. 107: 805-814.

III Kemmo, S., Ollilainen, V., Lampi A.-M. and Piironen, V. 2007. Determination of stigmasterol and cholesterol oxides using atmospheric pressure chemical ionization liquid chromatography/mass spectrometry. Food Chem. 101: 1438-1445.

IV Kemmo, S., Ollilainen, V., Lampi A.-M. and Piironen, V. 2007. Liquid chromatography mass spectrometry for plant sterol oxide determination in complex mixtures. Eur. Food Res. Technol. Online.

Contribution of the author to studies I-IV

I, III Suvi Kemmo planned the study together with the other authors. She was responsible for the experimental work and had the main responsibility for interpreting the results. She was the main author of the paper.

II Suvi Kemmo planned the study together with the other authors and performed part of the experimental work. She had the main responsibility for interpreting the results and was the main author of the paper.

IV Suvi Kemmo planned the study together with the other authors. She had the main responsibility for interpreting the results and was the main author of the paper.

LIST OF ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
BHT	butylated hydroxytoluene/2,6-di-t-butyl-p-cresol
BSTFA	N,O-bis-(trimethylsilyl)trifluoroacetamide
CID	collision-induced dissociation
CL	chemiluminescent detection/detector
CV	coefficients of variation
DPPP	diphenyl-1-pyrenylphosphine
EC (Hg)	mercury cathode electrochemical detection/detector
ELSD	evaporate light scattering detection/detector
ESI	electrospray ionization
FID	flame ionization detection/detector
FL	fluorescence detection/detector
GC	gas chromatography/chromatograph
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography/chromatograph
IUPAC-IUB	International Union of Pure and Applied Chemistry and International Union of Biochemistry
LC-MS	liquid chromatography-mass spectrometry
LDL	low-density lipoprotein
LLSD	laser light scattering detection/detector
$NaBH_4$	sodium borohydride
NaCl	sodium chloride
Na_2SO_4	sodium sulfate
NMR	nuclear magnetic spectroscopy
MeLo-OOH	methyl linoleate hydroperoxide
MeOH	methanol
MTBE	methyl-*tert*-butylether
PV	peroxide value
r	correlation coefficient
R^2	coefficient of determination
RI	refractive index detection/detector
RF	response factor
SIM	selective ion monitoring
SPE	solid-phase extraction
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
UV	ultraviolet detection/detector
QIT	quadropole ion-trap

CONTENTS

1. INTRODUCTION

The cholesterol-lowering effects of plant sterols have been known since the 1950s (Peterson et al., 1951; Pollak et al., 1953). Although many studies have been conducted in this area, the mechanism of cholesterol-lowering effect of plant sterols is not fully understood. It has been thought to involve the ability of plant sterols to prevent dietary cholesterol from being absorbed in the small intestine (Ostlund, 2002). In addition to the blood cholesterol-lowering effect, plant sterols have been suggested to possess anti-cancer, anti-atherosclerotic, anti-inflammatory and anti-oxidative effects in animals (Awad and Fink, 2000; Berger et al., 2004; Lagarda et al., 2006).

Plant sterols are not endogenously synthesized in the human body and are derived exclusively from dietary sources (Ling and Jones, 1995). The main sources of plant sterols in the diet are vegetable oils, margarines and cereal products (Piironen et al., 2000a; Wester, 2000; Weber and Mukherjee, 2006). The estimated daily dietary intakes of plant sterols among different populations range from 160 to 400 mg (Lagarda et al., 2006). The typical plant sterol or stanol intake required to obtain a 10-15% lowering in blood low-density lipoprotein (LDL) cholesterol is 1.5-3 g/day (Katan et al., 2003; Normèn et al., 2004). This can only be achieved by consumption of plant sterol-enriched food products, which are currently commercially available in many countries.

Like other unsaturated lipids, plant sterols and cholesterol are susceptible to oxidation when heated, exposured to ionizing radiation, light, chemical catalysts or enzymatic processes. After formation of initiating free radicals, the primary oxidation products of sterols, hydroperoxides, are formed. They decompose to secondary oxidation products such as hydroxy, epoxy and keto compounds. *In vitro* studies have demonstrated that cholesterol oxides may have cytotoxic, mutagenic and atherogenic activities, and thus, may have harmful effects on human health (Guardiola et al., 1996; Schroepfer, 2000). Several studies show that toxicity of the cholesterol oxidation products vary depending on their chemical structure (Brown and Jessup, 1999; Schroepfer, 2000). Due to their structural similarity, plant sterol oxidation products may have similar health implications. Results regarding potential toxic effects of plant sterol oxides are, however, limited and inconsistent. Meyer et al. (1998) and Adcox et al. (2001) observed toxic effects of some plant sterol oxides both *in vivo* and *in vitro*. Hiroko et al. (2004) and Lea et al. (2004), by contrast, reported no toxic effects of plant sterol oxides. Recently, biological effects of cholesterol and plant sterol oxidation products were compared and concluded to have qualitatively similar toxic effects; however higher concentrations of plant sterol oxides were required to achieve comparable levels of toxicity

(Maguire et al., 2003; Ryan et al., 2005). Because different oxides of plant sterols may have different toxicity effects (Ryan et al., 2005), it is important to develop methods to be able to follow the individual formation and decomposition profiles of oxides.

Foods contain a mixture of plant sterols and therefore oxidation leads to complex oxidation product mixtures. Analysis of plant sterol oxides has mainly been performed with gas chromatography (GC) techniques (Dutta, 1997; Dutta and Appelqvist, 1997; Lampi et al., 2002; Johnsson et al., 2003; Johnsson and Dutta, 2003; Apprich and Ulbert, 2004; Louter, 2004). GC methods require high temperatures and laborious sample preparation. Analysis of hydroperoxides is not possible with GC. High-performance liquid chromatography (HPLC) offers the advantage of operating under low temperatures, and thus, the analysis of thermolabile hydroperoxides is possible. In addition, less sample pretreatment is needed with LC methods. Thus far, only Giuffrida et al. (2004) have used HPLC for analysis of plant sterol oxides, however many researchers have analysed cholesterol oxides with HPLC methods (Kermasha et al., 1994; Razzazi-Fazeli et al., 2000; Mazalli et al., 2006). To be able to prevent oxidation, the formation patterns of individual primary and secondary oxidation products must be examined in detail.

This study concentrated on the development of HPLC methods to evaluate formation and further reactions of individual primary and secondary oxidation products of plant sterols. The applicability of the methods for following the oxidation reaction pathways of plant sterols at different temperatures and in different oxidation conditions was also investigated.

This thesis reviews the literature concerning structure, oxidation and analysis of sterols. The experimental data and the results published in studies (**I**-**IV**) are attached. The oxidation behaviour and methods developed are evaluated in the Discussion section.

2. REVIEW OF THE LITERATURE

2.1 Chemical structure of plant sterols

Plant sterols (phytosterols) are plant components with a similar chemical structure to cholesterol. Plant sterols belong to the group of triterpenes, and they may contain an extra methyl or ethyl group at C-24 and a double bond at C-22 compared with cholesterol (Figure 1). Plant sterols have a tetracyclic cyclopenta(α)phenantrene ring specific to steroids with methyl substitutions at C-10 and C-13, a hydroxyl group at C-3 and a flexible side-chain of varying length at C-17, as reviewed by many researchers (Goad, 1991; Hartmann, 1998; Piironen et al., 2000b). Plant sterols are present as mixtures in plants.

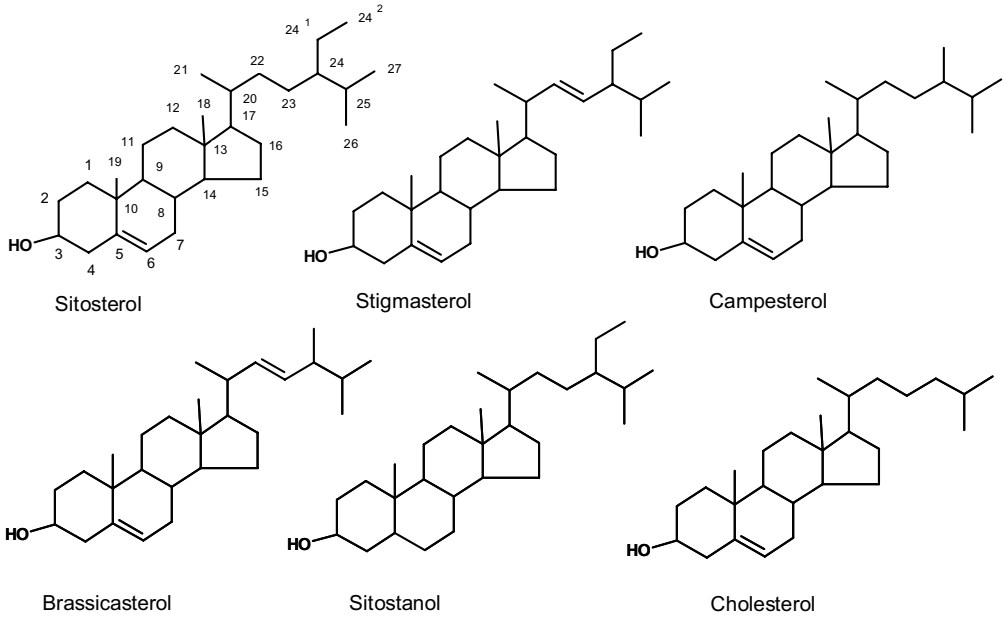

Figure 1. Chemical structures of the main sterols. Numbering is according to the International Union of Pure and Applied Chemistry and International Union of Biochemistry (IUPAC-IUB) (Moss, 1989).

Plants contain over 250 sterol and sterol-related compounds, as reported by Akihisa et al. (1991). Plant sterols can be divided on both a structural and a biosynthetic basis, into 4-desmethylsterols, 4-monomethylsterols and 4-dimethylsterols, which differ in the number of methyl groups at C-4 in the ring structure (Akihisa et al., 1991; Goad, 1991). 4-Dimethylsterol and 4-monomethylsterols are metabolic intermediates in the biosynthetic pathway leading to the end-products 4-desmethysterols. 4-Dimethylsterols and 4-monomethylsterols are usually present in low levels in most plant tissues (Piironen et al.,

2000b; Lagarda et al., 2006). 4-Desmethylsterols with no methyl substitution at C-4 are the major class of sterols in plants, and thus, in the human diet (Salo et al., 2003). The 4-desmethylsterols can also be categorized into Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$-sterols according to the position and number of double bonds in the ring structure (Nes, 1987). Most plant sterols are Δ^5 sterols (Moreau et al., 2002). Sitosterol is the main desmethylsterol in plants and is usually accompanied by its 22-dehydroanalogue, stigmasterol. Saturated plant sterols with no double bonds in their ring structure or side-chain are called stanols. Stanols occur at trace levels in many plant species and at high levels only in cereal species (Piironen et al., 2002). The amounts and relative proportions of different sterols in plants are dependent on the plant species.

In nature, plant sterols occur in five common lipid classes, i.e. as free alcohols, steryl fatty acid esters, hydroxycinnamate steryl esters, steryl glycosides, and esterified steryl glycosides (Figure 2) (Moreau, 2005). The last four forms are known as plant sterol conjugates.

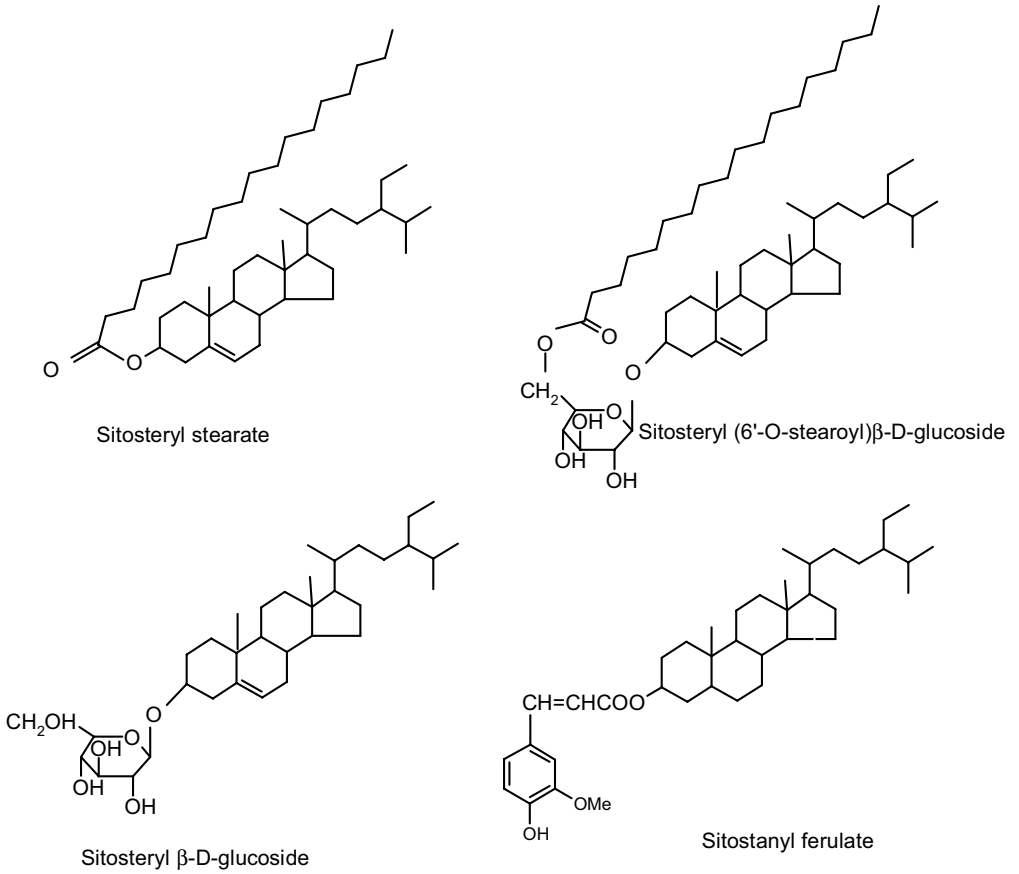

Figure 2. Examples of the main plant sterol conjugates.

2.2 Oxidation of sterols

The growing interest in producing plant sterol-enriched foods has activated research on the safety aspects of increasing sterol levels. The main questions to be studied include reactivity and stability of the added sterols. In the case of plant sterols, oxidation is the reaction of main concern. The first studies of the oxidation kinetics of free and esterified sitosterol were published in the 1980s (Yanishlieva and Marinova, 1980; Yanishlieva et al., 1983). Research on the oxidation of plant sterols has focused on analysis of the selected secondary oxidation products and the whole oxidation pathway, including formation and degradation of hydroperoxides and secondary oxidation products has not been investigated in detail. On the other hand, the oxidation of cholesterol has been more extensively studied. The oxidation of Δ^5-plant sterols has been suggested to follow the same scheme as cholesterol (Smith, 1987; Dutta and Savage, 2002).

Plant sterols are fairly stable molecules, but may undergo oxidation and several other transformation reactions in the presence of heat, light, metal contaminants and oxygen. Reactive oxygen species and oxidative enzymes may also induce plant sterol transformation. The oxidation products can be divided into two groups based on polarity of the compounds (Dutta and Savage, 2002). This study was focused on the oxidation products that are more polar than unoxidized sterol and have hydroperoxy, additional hydroxy, epoxy and keto groups in their structure. The non-polar compounds, which are less polar than unoxidized sterols, arise as a result of reactions occurring also at high temperatures such as dehydration or dehydrogenation. The nomenclature used for the major secondary oxidation products and some primary oxidation products of cholesterol, sitosterol, stigmasterol, campesterol and brassicasterol is presented in Table 1.

Table 1. Trivial and systematic names and commonly used abbreaviations of the main sterols and their polar oxidation products.

Systematic name	Trivial name	Abbreviation
cholest-5-en-3β-ol	**cholesterol**	**cholesterol**
3β-hydroxycholest-5-en-25-hydroperoxide	25-hydroperoxycholesterol	25-OOH-cholesterol
3β-hydroxycholest-5-en-5α-hydroperoxide	5α-hydroperoxycholesterol	5α-OOH-cholesterol
3β-hydroxycholest-5-en-6α-hydroperoxide	6α-hydroperoxycholesterol	6α-OOH-cholesterol
3β-hydroxycholest-5-en-6β-hydroperoxide	6β-hydroperoxycholesterol	6β-OOH-cholesterol
3β-hydroxycholest-5-en-7α-hydroperoxide	7α-hydroperoxycholesterol	7α-OOH-cholesterol
3β-hydroxycholest-5-en-7β-hydroperoxide	7β-hydroperoxycholesterol	7β-OOH-cholesterol
cholest-5-en-3β,25-diol	25-hydroxycholesterol	25-OH-cholesterol
cholest-4-en-3-one-6α-ol	6α-hydroxy-3-ketocholesterol	6α-OH-3-ketocholesterol
cholest-4-en-3-one-6β-ol	6β-hydroxy-3-ketocholesterol	6β-OH-3-ketocholesterol
5,6α-epoxy-5α-cholestan-3β-ol	cholesterol-5α,6α-epoxide	5α,6α-epoxycholesterol
5,6β-epoxy-5α-cholestan-3β-ol	cholesterol-5β,6β-epoxide	5β,6β-epoxycholesterol
3β-hydroxycholest-5-en-7-one	7-ketocholesterol	7-ketocholesterol
cholest-5-en-3β,6β-diol	6β-hydroxycholesterol	6β-OH-cholesterol
cholest-5-en-3β,7α-diol	7α-hydroxycholesterol	7α-OH-cholesterol
cholest-5-en-3β,7β-diol	7β-hydroxycholesterol	7β-OH-cholesterol
5α-cholest-3β,5α,6β-triol	cholestantriol	cholesterol-triol
(24R)-ethylcholest-5-en-3β-ol	**sitosterol**	**sitosterol**
(24R)-ethylcholest-4-en-3-one-6α-ol	6α-hydroxy-3-ketositosterol	6α-OH-3-ketositosterol
(24R)-ethylcholest-4-en-3-one-6β-ol	6β-hydroxy-3-ketositosterol	6β-OH-3-ketositosterol
(24R)-5α,6α-epoxy-24-ethylcholestan-3β-ol	sitosterol-5α,6α-epoxide	5α,6α-epoxysitosterol
(24R)-5β,6β-epoxy-24-ethylcholestan-3β-ol	sitosterol-5β,6β-epoxide	5β,6β-epoxysitosterol
(24R)-ethylcholest-5-en-3β-ol-7-one	7-ketositosterol	7-ketositosterol
(24R)-ethylcholest-5-en-3β,6β-diol	6β-hydroxysitosterol	6β-OH-sitosterol
(24R)-ethylcholest-5-en-3β,7α-diol	7α-hydroxysitosterol	7α-OH-sitosterol
(24R)-ethylcholest-5-en-3β,7β-diol	7β-hydroxysitosterol	7β-OH-sitosterol
(24R)-methylcholest-5-en-3β-ol	**campesterol**	**campesterol**
(24R)-methylcholest-4-en-3-one-6α-ol	6α-hydroxy-3-ketocampesterol	6α-OH-3-ketocampesterol
(24R)-methylcholest-4-en-3-one-6β-ol	6β-hydroxy-3-ketocampesterol	6β-OH-3-ketocampesterol
(24R)-5α,6α-epoxy-24-methylcholestan-3β-ol	campesterol-5α,6α-epoxy	5α,6α-epoxycampesterol
(24R)-5β,6β-epoxy-24-methylcholestan-3β-ol	campesterol-5β,6β-epoxy	5β,6β-epoxycampesterol
(24R)-methylcholest-5-en-3β-ol-7-one	7-ketocampesterol	7-ketocampesterol
(24R)-methylcholest-5-en-3β,6β-diol	6β-hydroxycampesterol	6β-OH-campesterol
(24R)-methylcholest-5-en-3β,7α-diol	7α-hydroxycampesterol	7α-OH-campesterol
(24R)-methylcholest-5-en-3β,7β-diol	7β-hydroxycampesterol	7β-OH-campeserol
(24S)-ethylcholest-5,22-dien-3β-ol	**stigmasterol**	**stigmasterol**
(24S)-ethylcholest-5,22-dien-3β-ol-25-hydroperoxide	25-hydroperoxystigmasterol	25-OOH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-5α-hydroperoxide	5α-hydroperoxystigmasterol	5α-OOH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-6α-hydroperoxide	6α-hydroperoxystigmasterol	6α-OOH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-6β-hydroperoxide	6β-hydroperoxystigmasterol	6β-OOH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-7α-hydroperoxide	7α-hydroperoxystigmasterol	7α-OOH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-7β-hydroperoxide	7β-hydroperoxystigmasterol	7β-OOH-stigmasterol

Table 1. Continued

Systematic name	Trivial name	Abbreviation
(24S)-ethylcholest-5,22-dien-3β-ol-7α-hydroperoxide	7α-hydroperoxystigmasterol	7α-OOH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-7β-hydroperoxide	7β-hydroperoxystigmasterol	7β-OOH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β,25-diol	25-hydroxystigmasterol	25-OH-stigmasterol
(24S)-ethylcholest-4,22-dien-3-one-6α-ol	6α-hydroxy-3-ketostigmasterol	6α-OH-3-ketostigmasterol
(24S)-ethylcholest-4,22-dien-3-one-6β-ol	6β-hydroxy-3-ketostigmasterol	6β-OH-3-ketostigmasterol
(24S)-5α,6α-epoxy-24-ethylcholest-22-en-3β-ol	stigmasterol-5α,6α-epoxide	5α,6α-epoxystigmasterol
(24S)-5β,6β-epoxy-24-ethylcholest-22-en-3β-ol	stigmasterol-5β,6β-epoxide	5β,6β-epoxystigmasterol
(24S)-ethylcholest-5,22-dien-3β-ol-7-one	7-ketostigmasterol	7-ketostigmasterol
(24S)-ethylcholest-5,22-dien-3β,6β-diol	6β-hydroxystigmasterol	6β-OH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β,7α-diol	7α-hydroxystigmasterol	7α-OH-stigmasterol
(24S)-ethylcholest-5,22-dien-3β,7β-diol	7β-hydroxystigmasterol	7β-OH-stigmasterol
(24S)-ethylcholest-22-en-3β,5α,7β-triol	stigmasteroltriol	stigmasterol-triol
(24S)-methylcholest-5,22-dien-3β-ol	**brassicasterol**	**brassicasterol**
(24S)-methylcholest-4,22-dien-3-one-6α-ol	6α-hydroxy-3-ketobrassicasterol	6α-OH-3-ketobrassicasterol
(24S)-methylcholest-4,22-dien-3-one-6β-ol	6β-hydroxy-3-ketobrassicasterol	6β-OH-3-ketobrassicasterol
(24S)-5α,6α-epoxy-24-methylcholest-22-en-3β-ol	brassicasterol-5α,6α-epoxide	5α,6α-epoxybrassicasterol
(24S)-5β,6β-epoxy-24-methylcholest-22-en-3β-ol	brassicasterol-5β,6β-epoxide	5β,6β-epoxybrassicasterol
(24S)-methylcholest-5,22-dien-3β-ol-7-one	7-ketobrassicasterol	7-ketobrassicasterol
(24S)methylcholest-5,22-dien-3β,6β-diol	6β-hydroxybrassicasterol	6β-OH-brassicasterol
(24S)methylcholest-5,22-dien-3β,7α-diol	7α-hydroxybrassicasterol	7α-OH-brassicasterol
(24S)methylcholest-5,22-dien-3β,7β-diol	7β-hydroxybrassicasterol	7β-OH-brassicasterol

2.2.1 Photo-oxidation

When oxidation of unsaturated fats is accelerated by exposure to light, oxidation is called photo-oxidation (Frankel, 1998). Plant sterols can be oxidized by photo-oxidation in the presence of oxygen, light energy and a photosensitizer. Photosensitizers absorb visible or near-UV light, to become electronically excited. There are two types of sensitizers that act with different mechanisms (Foote et al., 1991). Type I sensitizers, such as riboflavin, act as photochemically activated free radical initiators (Frankel, 1998). The sensitizer in the triplet state reacts with the lipid substrate by hydrogen atom or electron transfer to form radicals, which react with oxygen and form hydroperoxides. These hydroperoxides are the same as those formed in autoxidation. Type II sensitizers, such as chlorophyll and methylene blue, react with oxygen by energy transfer. Reactive singlet oxygen $^1O_2$ is generated, which reacts further with lipids and forms hydroperoxides. Due to the high energy of the singlet oxygen, the products formed differ from the products formed in autoxidation.

Singlet oxygen is very reactive and can react rapidly with double bonds in the steroid molecule by a specific ene-reaction (Kulig and Smith, 1973). When cholesterol is photo-

oxidized with a type II sensitizer, 5α-OOH-cholesterol is formed together with lower quantities of 6α-OOH- and 6β-OOH-cholesterol (Kulig and Smith, 1973; Smith, 1987; 1996; Korytowski et al., 1999; Min et al., 2002) which form due to the allylic shift of the Δ5 bond. 5α-OOH-cholesterol rearranges in non-polar dilute solutions to 7α-OOH-cholesterol, which is shown to epimerize to 7β-OOH-cholesterol (Smith et al., 1973; Teng et al., 1973; Beckwith, 1989; Porter et al., 1995; Smith, 1996; Adachi et al., 1998; Yoshida et al., 2003). However, such a rearrangement is a slow process. 7β-OOH-cholesterol and 7α-OOH-cholesterol are also formed when cholesterol is autoxidized. The different products formed during photo-oxidation compared with autoxidation enable cholesterol to be used as an indicator of photo-oxidation (Geiger et al., 1997; Yamazaki et al., 1999). Identification of 5α-OOH-, 6α-OOH- and 6β-OOH-cholesterol in a reaction system signifies that photo-oxidation has occurred.

Information about plant sterol photo-oxidation is limited. Bortolomeazzi et al. (1999) photo-oxidized sitosterol, campesterol, stigmasterol and brassicasterol by using hematoporphyrine as a sensitizer. They observed the formation of 5α-OOH-sterol as a main product together with small amounts of 6α-OOH- and 6β-OOH-sterols. 7α-OOH- and 7β-OOH-sterols were also obtained by allowing the 5α-OOH-sterol to rearrange to the 7α-OOH-sterol, which in turn epimerized to the 7β-OOH-sterol (Bortolomeazzi et al., 1999).

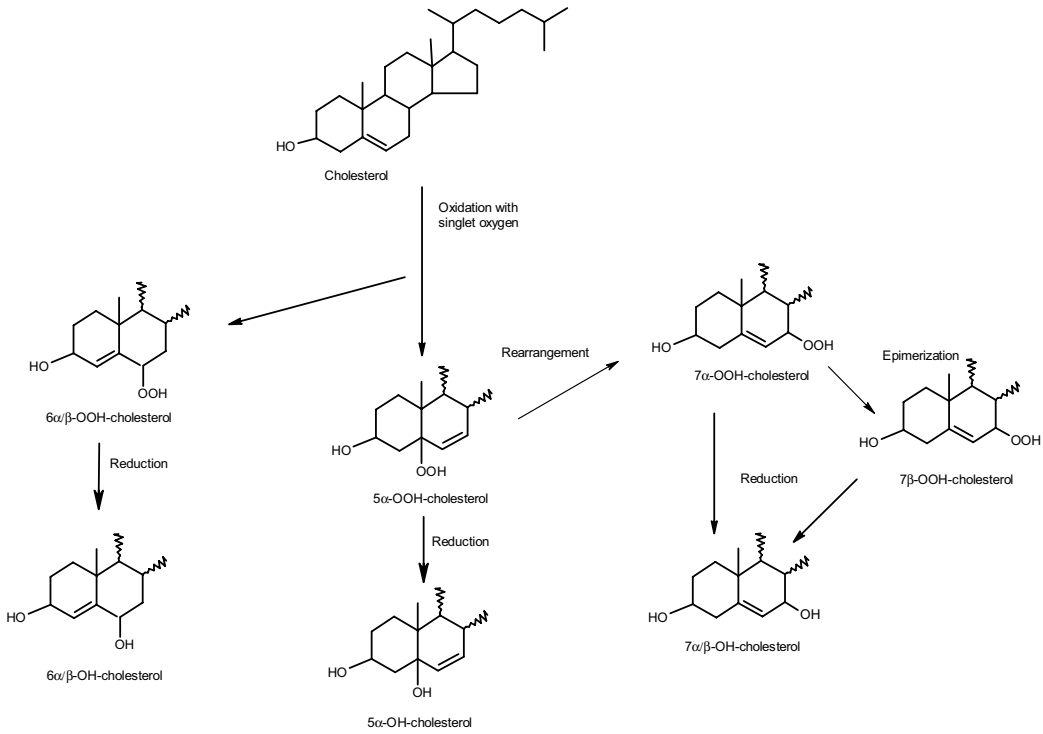

Figure 3. Formation of cholesterol oxidation products during photo-oxidation (Smith, 1996).

2.2.2 Autoxidation and thermo-oxidation

Autoxidation is a free radical chain process containing initiation, propagation and termination steps (Figure 4) (Porter et al., 1995; Frankel, 1998). The rate of autoxidation is increased with an increasing number of double bonds in the lipid molecule. Autoxidation of sterols follows a similar basic oxidation pathway as that of monounsaturated fatty acids. Autoxidation of plant sterols has been proposed to follow the same scheme as the extensively researched autoxidation of cholesterol. In this study, autoxidation at high temperatures is called thermo-oxidation. At high temperatures, other reactions, such as dehydration, dehydrogenation and polymerization, become more important.

Initiation:	$In^{\bullet} + RH \Rightarrow InH + R^{\bullet}$
Propagation:	$R^{\bullet} + O_2 \Rightarrow ROO^{\bullet}$
	$ROO^{\bullet} + RH \Rightarrow R^{\bullet} + ROOH$
Termination:	$2ROO^{\bullet} \Rightarrow$ non-radical products

Figure 4. Free radical autoxidation. In = initiator, R = lipid moeity, • = radical.

Formation of main oxidation products

Autoxidation of cholesterol occurs in a solid state, in solutions or in aqueous dispersions in the presence of molecular oxygen. The first free radicals formed when cholesterol is oxidized by autoxidation seem to arise from abstraction of hydrogen atoms from carbons C-7 and C-25 (Smith, 1996; Blekas and Boskou, 1999; Lercker and Rodriguez-Estrada, 2002) (Figure 5). The primary product, 7-hydroperoxide, is formed after an allylic hydrogen atom has been abstracted from C-7, forming a free radical. The reaction is followed by attack of molecular triplet oxygen ($^3O_2$), yielding 7-peroxyl radicals (Lercker and Rodriguez-Estrada, 2002; Dutta, 2004). 7-peroxyl radicals are stabilized, in turn, by hydrogen, and 7α-OOH- and 7β-OOH-cholesterol are thus formed. They are the only cholesterol hydroperoxides that remain stable at room temperature (Lercker and Rodriguez-Estrada, 2002). 7α-OOH- and 7β-OOH-cholesterol are further decomposed to more stable 7α-OH- and 7β-OH-cholesterol. The formation of 7-ketocholesterol can be attributed to dehydration of 7-OOH-cholesterol or dehydrogenation of 7-OH-cholesterol under dry and oxygen-rich conditions (Teng et al, 1973; Smith, 1987; Kim and Nawar, 1993; Chien et al., 1998).

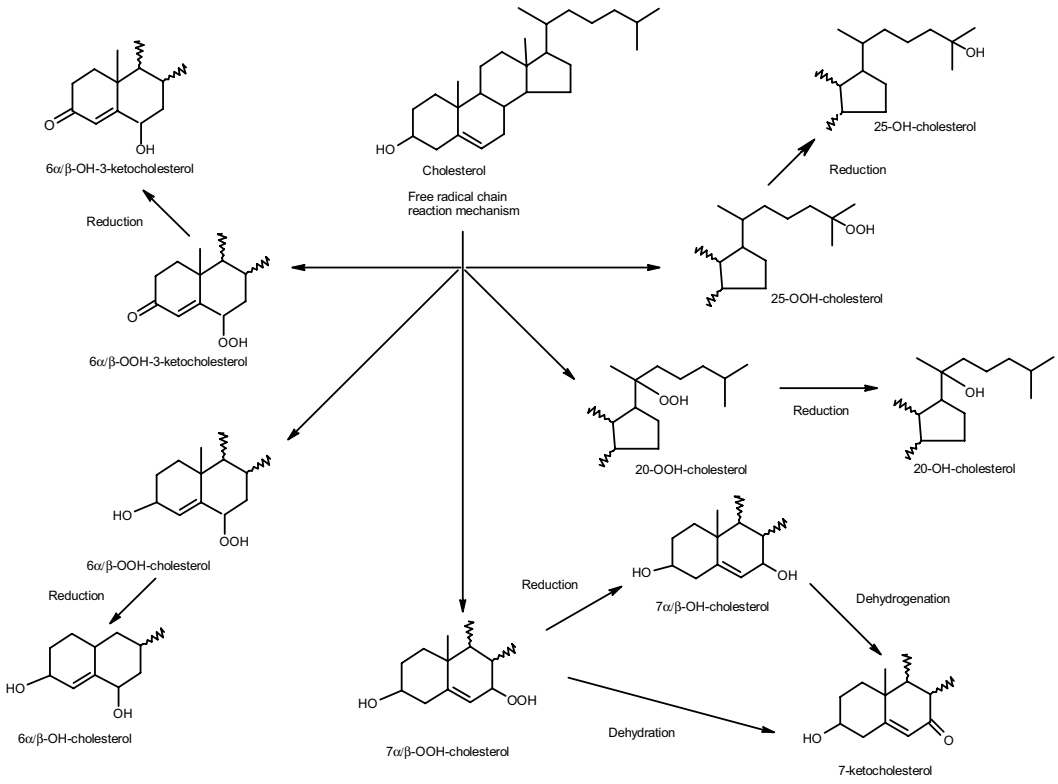

Figure 5. Formation of cholesterol oxides with autoxidation (Smith, 1987).

Formation of epoxides with autoxidation occurs by a bimolecular reaction using radical addition mechanism (Figure 6). The unoxidized sterol molecule reacts with a peroxyl radical, and α-epoxides and β-epoxides are formed (Smith, 1987; 1996; Lercker and Rodriguez-Estrada, 2002; Dutta, 2004). Formation of 5β,6β-epoxycholesterol is more favourable than formation of 5α,6α-epoxycholesterol (Lercker and Rodriguez-Estrada, 2002). This might be due to steric hindrance of the OH group at position 3. The triols can be formed from both epimers of 5,6-epoxides through hydration in an acidic environment. Autoxidation by elevated temperature does not generate any triols.

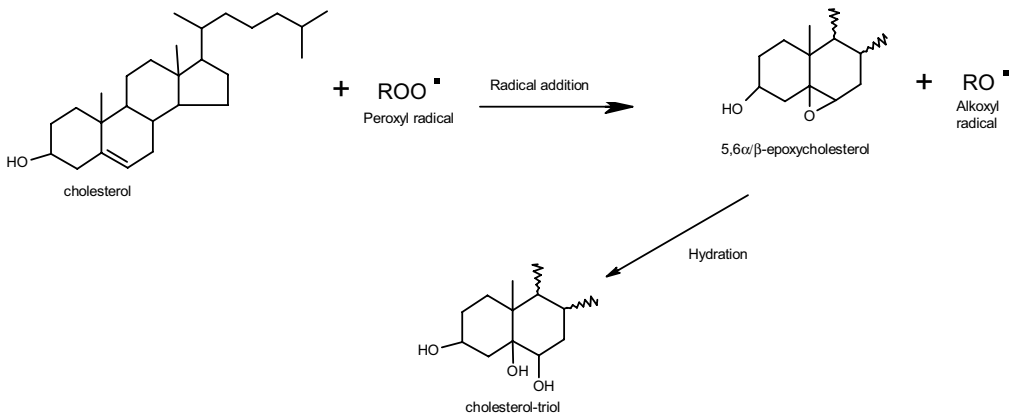

Figure 6. Formation of cholesterol epoxides and triol (Lercker and Rodriguez-Estrada, 2002).

Formation of side-chain hydroperoxides

Oxygen can also attack the side-chain tertiary carbons, leading to formation of 20-OOH- and 25-OOH-cholesterol. In the case of plant sterols 24-OOH-sterol can also be formed. These hydroperoxides decompose to more stable corresponding hydroxides (Figure 5). However, oxidation of the steroid side-chain is reported to be much less pronounced than oxidation in the ring structure. These side-chain autoxidation products are generally formed in the solid state, but not in dispersions or solutions (Korahani et al., 1982; Smith, 1987).

Other reactions occurring at high temperatures

At high temperatures, thermoreactions such as dehydrogenation and dehydratation become more important. One independent mechanism, especially at high temperatures, is the formation of 6α,β-OOH-3-ketocholesterol (Figure 5). First 3β-alcohol is dehydrogenated to cholest-5-en-3-one. This is rearranged to cholest-4-en-3-one and is then oxygenated to epimeric 6α,β-OOH-3-ketocholesterol (Korahani et al., 1982; Smith, 1987). The 6α,β-OOH-3-ketocholesterol, in turn, decomposes to corresponding 6α,β-OH-3-ketocholesterol, cholest-4-en-3,6-dione and 5α-cholestane-3,6-dione. When cholesterol or its oxidation products are exposed to heating, less polar products, such as conjugated diene cholest-3,5-diene, conjugated triene cholesta-3,5-dien-7-one, cholesta-5,7-dien-3β-ol and cholesta-3,5,7-triene, are also formed with a dehydration reaction (Lercker and Rodriguez-Estrada, 2002). When heating temperature is over 170°C, 3,3-dicholesteryl ether is produced with dehydration following a bimolecular reaction mechanism.

Conditions affecting oxidation product profiles

Oxidation temperature, oxidation time, presence of water and buffer have been observated to have effects on the products formed and their relative amounts (Kim and Nawar, 1993). Cholesterol is fairly stable during heating at 100°C, but becomes unstable at temperatures above 120°C (Osada et al., 1993). Composition of the oxides differs depending on temperature and time of heating. Chien et al. (1998) studied the kinetic model of cholesterol oxidation during heating at 150°C for 30 min. The highest rate constant was observed for 7-OOH-cholesterol formation, followed by epoxidation, dehydration, reduction and dehydrogenation. They also noted that dehydration was more prone to happen than reduction under dry conditions. The formation rate of 7-ketocholesterol was shown to increase with increasing 7-OH-cholesterol concentration. 7β-OOH-cholesterol was more labile towards thermal decomposition than 7α-OOH-cholesterol (Teng et al., 1973). The 7β-OH-cholesterol was predominant, possibly due to greater thermodynamic stability of the equatorial 7β-OH-cholesterol as compared with the axial 7α-OH-cholesterol (Paniangvait et al., 1995).

Oxidation reactions of plant sterols

The autoxidation mechanism of plant sterols has not been investigated as extensively as that of cholesterol. However, available information suggests that plant sterols are oxidized in the same manner as cholesterol. Yanishleava et al. (1980; 1983) were the first to evaluate the oxidation kinetics of free and esterified sitosterol at different temperatures. They noted that when free sitosterol and sitosteryl stearate were oxidized the main products formed were 7α-OOH and 7β-OOH. In the case of free sterol, 6β-OOH-3-ketositosterol was also one of the main peroxy products formed together with sitosta-4-en-3-one and sitosta-4-en-3,6-dione. Daly et al. (1983) heated β-sitosterol at 100°C for 48 h and identified formation of polar 7α- and 7β-OH-sitosterol, 7-ketositosterol and 5α,6α- and 5β,6β-epoxysitosterol and non-polar sitosta-4-en-3-one, sitosta-4-en-3,6-dione and sitosta-5-en-3-one. Gordon and Magos (1984) identified most of the same products during heating of cholesterol and Δ^5-avenasterol at 180°C for 72 h. Recently, Zhang et al. (2005) studied formation of sitosterol oxides when heating pure sitosterol at 100°C, 150°C and 200°C. They found no detectable level of oxides at 100°C in 30 min. At 150°C and 200°C, the main sitosterol oxides present during heating were 7α-OH-, 7β-OH, 5α,6α-epoxy, 5β,6β-epoxy and 7-ketositosterol. Sitosterol-triol was not observed. The major oxide formed at both temperatures was 7-ketositosterol. They also noted that the formation rate of oxides was quite constant during the first 30 min of heating at 150°C. By contrast, at 200°C, the oxide content increased very quickly during the first 15 min, slightly decreasing after 20-25 min (Zhang et al., 2005). Similar results have been

described for cholesterol by Osada et al. (1993). Johnsson and Dutta (2003) reported the formation of side-chain and other oxidation products, such as 24-OH-sitosterol, 25-OH-sitosterol, 6α-OH-3-ketositosterol, 6β-OH-3-ketositosterol, 24-OH-campesterol, 25-OH-campesterol, 6α-OH-3-ketocampesterol and 6β-OH-3-ketocampesterol, when commercial mixture of sitosterol and campesterol was heated for 72 h at 120°C. The 25-OH-stigmasterol and 24-OH-stigmasterol were formed together with common ring structure oxidation products when stigmasterol was oxidized at 120 °C for 72 h (Johnsson et al., 2003).

2.3 Formation of plant sterol oxides in food models and foods

Oxidation of plant sterols in food models

An interesting interaction between plant sterols and lipid matrices has been observed by many researchers (Blekes and Boskou, 1989; Lampi et al., 2000; Oehrl et al., 2001; Soupas et al., 2004a; 2007). Blekes and Boskou (1989) investigated the products formed when a triacylglycerol mixture containing 5% stigmasterol was heated at 180°C for 16 h. They identified non-polar products stigmasta-3,5,22-triene, stigmasta-3,5,22-trien-7-one and stigmasta-4,22-dien-3-one as well some more polar compounds. Lampi et al. (2000) also investigated the stability of plant sterols in oils during heating at various temperatures. They added sitosterol, stigmasterol, sitostanol and ergosterol and cholesteryl oleate to rapeseed oil and tripalmitin at a level of 0.1-1%. They noted that sitostanol, with a saturated ring structure, was more stable than other sterols, while ergosterol, $\Delta^{5,7}$-sterol, was the least stable in both oils. They also observed that heating temperature had a significant effect on the stability of sterols. All sterols were relatively stable at 80°C, while significant losses occurred already at 120°C. All sterols were more stable in rapeseed oil than tripalmitin at 180°C. On the other hand, Oehrl et al. (2001) studied the oxidative stability of plant sterols in canola, coconut, peanut and soybean oils and found that sterol losses were greater in canola and soybean oils than in more saturated oils. A greater variety of plant sterol oxides was observed at the lower temperatures of 100°C and 150°C as compared with 180°C. At 150°C and 180°C, decomposition of plant sterol oxides occurred (Oehrl et al., 2001). Soupas et al. (2004a) heated stigmasterol and sitostanol at different temperatures in different lipid matrices for various periods of time. Their results showed that temperature (60-180°C), heating time (0.5-8 weeks), sterol structure and lipid matrix composition (purified rapeseed oil and tripalmitin) influenced oxidation. Interactions between lipid matrices and temperatures particularly appeared to have important effect on total oxides formed and reaction pathways of oxidation. At high temperatures, sterols were more stable in an unsaturated matrix than in a saturated one, but at temperatures under 140°C this situation was reversed (Soupas et al., 2004a). They

also investigated how esterification of sitosterol, campesterol and sitostanol with rapeseed oil fatty acids affects oxidation reactions. In addition, they examined interactions between plant sterol/stanol and saturated lipid matrix at 100°C and 180°C. The results revealed that free and esterified plant sterols differed in reactivity. Plant sterol esters were more reactive than free sterols during heating at 100°C. In contrast, free sterols were slightly more reactive than esters at 180°C. The oxidation of plant stanol compounds was low under all conditions used (Soupas et al., 2005). Recently, Soupas et al. (2007) evaluated oxidative stability of different sterol compounds during pan-frying. The lipid matrices used were rapeseed oil, rapeseed oil-based liquid margarine and butter oil. Pan-frying seemed to induce plant sterol oxidation, but had no marked effect on plant stanol oxidation; up to 5.1% of initial sitosterol and 0.1% of stanol were found as oxides. With higher frying temperature and longer frying time, more oxides were formed. A saturated lipid matrix and the presence of water accelerated oxidation under pan-frying conditions (Soupas et al., 2007).

Occurrence of plant sterol oxides in foods

Data on the levels of plant sterol oxides in foods are very limited. The evaluation of plant sterol oxides in foods is difficult because of the lack of commercial standards and great number of compounds formed during oxidation of plant sterol mixtures. Quantification of plant sterol oxides in foods is also challenging because of the large amounts of interfering compounds such as unoxidized plant sterols and other lipids.

Small amounts of plant sterol oxides have been observed in some food products, i.e. French fries, potato chips (Dutta, 1997), wheat flour (Nourooz-Zadeh and Appelqvist, 1992), plant sterol- enriched whole milk powder and heat-treated milk (Soupas et al., 2006), commercial margarine (Conchillo et al., 2005), plant sterol-enriched margarine (Grandgirard et al., 2004a; Conchillo et al. 2005) and vegetable oils (Zhang et al., 2005) (Table 2). The measured plant sterol oxides in different vegetable oils, French fries and potato chips were 7α-OH-, 7β-OH-, 7-keto-, 5α,6α-epoxy-, 5β,6β-epoxy and triol compounds of sitosterol and campesterol (Dutta, 1997; Dutta and Appelqvist, 1997). Wheat flour samples stored different times contained 5α,6α-epoxy-, 5β,6β-epoxy, 7α-OH- and 7β-OH-sitosterol (Nourooz-Zadeh and Appelqvist, 1992). The only oxides that were over the detection limit throughout the storage period in enriched milk powder were 7α-OH-sitosterol and 7β-OH-sitosterol (Soupas et al. 2006). The heat-treated (127°C, 2 s) non-fat milks enriched with free or esterified plant sterols (78% sitosterol) or with plant stanyl esters contained only low levels of oxides. The major products in these milk samples were 7α-OH-, 7β-OH- and 7-ketositosterol. Conchillo et al. (2005) observed that for all plant sterols the main oxidation products were the 7-keto derivatives in

both non-enriched and enriched margarines. Zhang et al. (2005) quantified and identified 7α-OH-, 7β-OH-, 5α,6α-epoxy, 5β,6β-epoxy-, 7-ketositosterol and sitosterol triol in sunflower and olive oil before and after heating at 150°C and 200°C for 60 min. They observed that the main oxides were the same at both temperatures. At 150°C, 7β-OH-sitosterol and 7-ketositosterol were the main products, while at 200°C 7-ketositosterol was predominant, followed by 5β,6β-epoxysitosterol.

Table 2. Occurrence of plant sterol oxides in foods.

Food	Analysis method	Total amount of plant sterol oxides (µg/g)	Reference
Wheat flour two months old	GC-FID	35*, 328*, I	Nourooz-Zadeh and Appelqvist, 1992
Rapeseed oil/palm oil blend (a) Sunflower oil (b) High-oleic sunflower oil (c) French fries fried at 200°C in oil a, in oil b, in oil c	GC-FID	41 , 60II 40, 57II 46, 56II 32* 37* 54*	Dutta , 1997
Potato chips fried in palm oil, in sunflower oil, in high-oleic sunflower oil	GC-FID	5* , 9*,III 46*, 47*III 35*, 58*, III	Dutta and Appelqvist, 1997
Commercial spread enriched with 8% plant sterol mixture	GC-FID	68	Grandgirard et al., 2004a
Non-enriched commercial spread (0.3% plant sterols) Enriched spread (6% plant sterols)	GC-FID	13 46	Conchillo et al., 2005
Sunflower oil Olive oil	GC-MS	50, 815 IV -, 365 IV	Zhang et al., 2005
Sitosterol-enriched milk powder Plant sterol-enriched heat-treated milk Plant sterol ester-enriched heat-treated milk Plant stanol ester-enriched heat-treated milk	GC-FID and GC-MS	14, 19V, 34VI 2 2 0.2	Soupas et al., 2006

* Total amount of plant sterol oxides in the product lipids, I Total amount of oxides after 36 months of storage, II after two days of frying, III after 25 weeks of storage, IV after heating at 200°C for 60 min, V after 12 months at room temperature, VI after 12 months at 38°C.

2.4 Analysis of sterol oxidation products

Analysis of plant sterol oxides is challenging. Many plant sterols occur simultaneously in foods and in ingredients used in sterol enrichment, and their oxidation thus leads to complex oxidation product mixtures. Artefact formation and losses during sample collection, storage and sample pretreatment can also be a problem, as reported by many researchers (Park et al., 1996; Smith, 1996; Lampi et al., 2002). Generally, methods used for plant sterol oxide analysis are based on those developed for cholesterol oxides including saponification of the sample, extraction of unsaponifiables, enrichment of the oxidation products, and chromatographic analysis mainly with GC-flame ionization detection (GC-FID) or GC-mass spectrometry (GC-MS). The quantification is usually performed with internal standard or external standard methods using commercially available cholesterol oxides. By GC methods mainly internal standard methods are used. Instead with LC methods quantification with external standards is generally used (Guardiola et al., 2004). GC methods require time-consuming sample preparation and high temperatures. Analysis of thermolabile hydroperoxides is not possible with GC. To prevent and control oxidation, the whole oxidation chain must be studied, including formation and decomposition of thermolabile hydroperoxides and also secondary oxidation products. The development of new methods for studying oxidation pathways of plant sterols is therefore needed.

2.4.1 Plant sterol oxide analysis with GC

Plant sterol oxides are usually analysed in foods and food models with GC-FID and GC-MS methods (Table 2) (Dutta, 1997; Dutta and Appelqvist, 1997; Lampi et al., 2002; Soupas et al., 2002; Johnsson and Dutta, 2003; Johnsson et al., 2003; Soupas et al., 2003; Apprich and Ulberth, 2004; Grandgirard et al., 2004a; 2004b; Johannes and Lorenz, 2004; Soupas et al., 2004a; 2004b; 2005). GC has an advantage over LC in that it has a much better separation power and can use universal FID. However, GC techniques require elevated temperatures, which can cause artefacts and makes direct analysis of thermolabile hydroperoxides impossible. In addition, derivatization of sterol oxides to trimethylsilyl (TMS) ether derivatives is needed to make the sterol oxides more volatile (Burkard et al., 2004; Louter et al., 2004; Raith et al., 2005), which increases the analysis time. If hydroperoxides are analysed with GC techniques, reduction of hydroperoxy group to the corresponding hydroxy group is required (Ohshima et al., 1996).

With GC-FID, coelution of non-oxidized and oxidized sterols may be a problem, as reported previously (Lampi et al., 2002; Soupas et al., 2004b). GC-MS, by contrast, allows

identification and quantification of partially or totally overlapped peaks. Moreover, sensitivity of GC-MS methods is similar or significantly better than that of GC-FID methods when operated in a selected ion mode (Guardiola et al., 2004). When TMS derivatization is used, less extensive fragmentation is obtained and compounds are easier to identify. In GC-FID or GC-MS analysis, quantification is usually made with corresponding cholesterol oxides (Plat et al., 2001) because plant sterol oxides are not commercially available. A general relative response factor 1 is usually used in GC-FID (Lampi et al., 2002). Recently, indirect quantification via GC-FID was used in GC-MS analysis of plant sterol oxides by Soupas et al. (2004a).

2.4.2 Sterol oxide analysis with HPLC

2.4.2.1 Separation and detection of sterol oxides in general

The major advantage offered by HPLC compared with GC is operation at room temperature, making analysis of thermolabile hydroperoxides possible. Thus, investigating the whole oxidation chain of plant sterols is possible with HPLC. HPLC can be used in normal phase (NP) and reverse phase (RP) modes, and it can be coupled to a wide range of detectors. There is also a possibility to connect two detectors online, which increases the use of this chromatographic technique. LC methods used for sterol oxide analysis are presented in Table 3.

Both NP and RP chromatography has been used for HPLC analysis of cholesterol oxides (Guardiola et al., 2004). In general, NP-LC systems have proven more effective than RP, but full resolution over the entire polarity range of the oxidation products was not achieved with isocratic conditions (Careri et al., 1998; Manini et al., 1998; Guardiola et al., 2004). With a NP column, the positional and epimeric isomers of cholesterol oxides could be separated. Less polar compounds than cholesterol could be separated with a column packed with alumina and silica (Lakritz and Jones, 1997; Guardiola et al., 2004). With a cyanopropyl column (CN column), the common B-ring oxides, side-chain oxides and also triol could be separated using an isocratic elution system. C18 columns are the most widely used columns for RP separation. The size and alkyl group-specific RP columns require less equilibration time and are less sensitive to moisture than NP columns. On the other hand, they are not as effective as NP columns in separating positional and epimeric isomers of sterol oxides.

Ultraviolet detection (UV) is the most commonly used method when cholesterol oxides are analysed with LC. In the case of cholesterol, a large diversity of oxides are formed, which

have their maximum UV absorption at different wavelengths (Kermasha et al., 1994; Caboni et al., 1997; Guardiola et al., 2004). Hydroxycholesterols have their absorption maximum around 205 nm, 7-ketocholesterol at 233-245 nm and conjugated non-polar triens at 280 nm (Osada et al., 1999). However, the most generally used wavelength is between 205 and 210 nm. There is also some cholesterol oxidation products such as 5α,6α-epoxy- and 5β,6β-epoxy-cholesterol and cholesterol-triol that do not have double bonds and therefore have inadequate UV absorption (Kermasha et al., 1994; Caboni et al., 1997; Careri et al. 1998; Manini et al., 1998; Csallany et al., 1989; Osada et al., 1999; Razzazi-Fazeli et al., 2000; Mazalli et al., 2006). The refractive index (RI) detector (Chen B and Chen Y, 1994), FID (Maerker et al., 1988) light scattering detector (LSD) (Kermasha et al., 1994; Caboni et al., 1997), evaporative light scattering (ELSD) (Lakritz and Jones, 1997) and UV-RI (Mazalli et al., 2006) have been used for the detection of cholesterol oxides. With LSD, ELSD and RI detectors, the analysis of 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol and cholesterol-triol is possible (Chen B and Chen Y, 1994; Kermasha et al., 1994; Caboni et al., 1997; Lakritz and Jones, 1997). The RI detector is less sensitive than UV detection and is not suitable for gradient elution (Chen B and Chen Y, 1994). The sensitivity of LSD is better than RI and similar to UV, except in the case of 7-ketocholesterol, where it was about 10 times less sensitive than UV (Kermasha et al., 1994; Caboni et al., 1997). With ELSD, all solutes less volatile than the solvent can be analysed and the detector response is based on molecular mass. The disadvantage of ELSD is the limited range of linear response (Lakritz and Jones, 1997).

Table 3. HPLC methods used for sterol oxide analysis

Method	Column [mobile phase]	Food/matrix	Oxidation products analysed	Reference
HPLC-FID	Silica column (300x3.9 mm, 10 µm) [hexane/isopropanol, gradient]	-	Cholest-3,5-dien-7-one; cholesterol; 25-OH-cholesterol; 5α,6α-epoxycholesterol; 5β,6β-epoxycholesterol; 6-ketocholesterol; 7-ketocholesterol; 7α-OH-cholesterol; 7β-OH-cholesterol; cholesterol-triol	Maerker et al., 1988
HPLC-UV (206 nm, 233 nm)	Silica column (300x3.9 mm, 10 µm) [hexane/isopropanol 93:7 v/v, isocratic]	Pork muscle, mouse liver tissues	25-OH-cholesterol; 7-ketocholesterol; 7β-OH-cholesterol; 7α-OH-cholesterol	Csallany et al., 1989
HPLC-UV (212 nm, 234 nm, 280 nm)	CN column (250x46 mm, 5 µm) [hexane/isopropanol 95:5 v/v, isocratic] C18 column (250x46 mm,5 µm) [acetonitrile/methanol, 55:45 v/v, isocratic]	Heated lard	Cholest-3,5-dien; cholest-4,6-dien-3-one; 25-OH-cholesterol; 5α,6α-epoxycholesterol; 5β,6β-epoxycholesterol; 6-ketocholesterol; 7-ketocholesterol; 7β-OH-cholesterol; cholesterol-triol	Chen and Chen, 1994
HPLC-UV (206 nm, 233 nm), HPLC-LSD*	Silica column (300x3.9 mm, 10 µm) [hexane/isopropanol, gradient]	-	20-OH-cholesterol; 25-OH-cholesterol; 7-ketocholesterol; 7α-OH-cholesterol; 7β-OH-cholesterol; 5α,6α-epoxycholesterol*; 5β,6β-epoxycholesterol*; cholesterol-triol*	Kermasha et al., 1994
HPLC-UV (205 nm)	Silica column (100x4.6 mm, 3 µm) [hexane/isopropanol/acetonitrile 95.8:3.90:0.30 (v/v/v), isocratic]	Low density lipoprotein (LDL)	7α-OOH-cholesterol; 7β-OOH-cholesterol; 7-ketocholesterol; 7α-OH-cholesterol; 7β-OH-cholesterol	Brown et al., 1997
HPLC-UV (210 nm) HPLC-LSD*	CN column (250x46 mm, 5 µm) [hexane/ethanol 97:3 v/v, isocratic]	Egg yolk powder	20α-OH-cholesterol; 25-OH-cholesterol; 19-OH-cholesterol; 5α,6α-epoxycholesterol*; 5β,6β-epoxycholesterol*; 7-ketocholesterol; 4β-OH-cholesterol; 7β-OOH-cholesterol; 7α-OOH-cholesterol; 7β-OH-cholesterol; 7α-OH-cholesterol; cholesterol-triol*	Caboni et al., 1997
HPLC-ELSD	Alumina/silica column (250x46 mm, 5 µm) with quard silica column (20x2 mm) [dichloromethane/acetonitrile/methanol, gradient]	-	Cholest-4-ene-3,6-dione; cholest-4-en-3-one; cholest-4,6-dien-3-one; cholesterol; 20α-OH-cholesterol; 7-ketocholesterol; 5α,6α-epoxycholesterol; 5β,6β-epoxycholesterol; 6-ketocholesterol; 7β-OH-holesterol; 7α-OH-cholesterol	Lakritz and Jones, 1997

Table 3. Continued

Method used	Column [mobile phase]	Food/matrix	Oxidation products analysed	Reference
HPLC-mercury cathode electrochemical detection (EC (Hg))	C18 (70x4.6 mm,3 µm) [methanol,/acetonitril,/isopropanol/ aqeous solution with 10mM ammonium acetate and 1mM sodium perchlorate 72:11:8:9 v/v, isocratic]	Mammalian cell	5α-OOH-cholesterol; 6α-OOH-cholesterol; 6β-OOH-cholesterol; 7α-OOH-cholesterol; 7β-OOH-cholesterol	Geiger et al., 1997
HPLC-chemiluminescent detection (CL)[1], HPLC-MS[2]	C8 (150x4.6 mm,) [methanol/water/acetonitril 89:9:2 v/v, isocratic][1] C18 (250x4.6 mm, 5µm) [methanol or methanol with 0.1M ammonium acetate, isocratic][2]	Erythrocyte membrane	7α-OOH cholesterol; 7β-OOH-cholesterol	Adachi et al., 1998
HPLC-MS (particle beam, quadrupole)	CN column (250x2.0 mm, 5 µm) [heptane/isopropanol 94:6 v/v, isocratic] ODS column (250x2.0 mm, 5 µm) [methanol/acetonitrile 90:10 v/v, isocratic]	-	Cholesterol; 5α,6α-epoxycholesterol; 25-OH-cholesterol; 7-ketocholesterol; 7β-OH-cholestrol; cholesterol-triol	Careri et al., 1998
HPLC-RI	CN column (250x4.6 mm, 5 µm) [hexane/isopropanol 95:5 v/v, isocratic]	-	5α,6α-epoxycholesterol; 5β,6β-epoxycholesterol; 7-ketocholesterol; 7α-OOH-cholesterol; 7β-OOH-cholesterol; 7α-OH-cholesterol; 7β-OH-cholesterol	Chien et al., 1998
HPLC-MS (APCI, quadrupole mass)	C18 (250x4.6 mm,5 µm) [methanol/acetonitrile 90:10 v/v, isocratic]	Beef	Cholesterol; 25-OH-cholesterol; 5α,6α-epoxycholestrol; 7-ketocholesterol; cholesterol-triol	Manini et al., 1998
HPLC-CL	Chiral phase column (250x4.6 mm) [methanol/water 85:15 v/v, isocratic]	Rats skin	5α-OOH-cholesterol	Yamazaki et al., 1999

Table 3. Continued

Method used	Column [mobile phase]	Food/matrix	Oxidation products analysed	Reference
HPLC-UV (205 nm, 234 nm and 280 nm) HPLC-ELSD	C18 (250x4.6 mm, 5 µm) [methanol/acetonitrile 60:40 v/v]	Standards, heated tallow, photo-oxidized cholesterol	3,5-cholestadien-7-one, 4,6-cholestadien-3-one, 5α-cholestan-3,6-dione, 20α-OH-cholesterol, 25-OH-cholesterol, 26-OH-cholesterol, 6-ketocholesterol, 7-ketocholesterol, 7β-OH-cholesterol, 7α-OH-cholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, cholesterol-triol"	Osada et al., 1999
HPLC-MS	C18 (250x4.6 mm, 5 µm) [methanol or methanol with 0.1M ammonium acetate, isocratic]	Human liver tissues	7-OOH-cholesterol, 7-ketocholesterol, 7-OH-cholesterol, 5β,6β-epoxycholesterol	Adachi et al., 2000
HPLC-MS (APCI, quadru-pole mass)	C18 (250x4.6 mm, 5 µm) [acetonitrile/methanol 60:40 v/v, isocratic]	Butter, butter oil, lard egg powder	25-OH-cholesterol,5α,6α-epoxy-cholesterol, 5β,6β-epoxy-cholesterol, 7-ketocholesterol, 7α-OH-cholesterol, 7β-OH-cholesterol, cholesterol-triol	Razzazi-Fazeli et al., 2000
HPLC-MS (APCI, triple quadrupole)	C18 (125x2.0 mm, 5 µm) with guard column (4x8 mm) [methanol/acetonitrile /10mM ammonium acetate buffer (PH 4.5) gradient]	Plasma	24S-OH-cholesterol, 27-OH-cholesterol	Burkard et al., 2004
HPLC-MS (ESI, triple quadrupole)	C18 (250x2.1 mm, 5 µm) [acetonitril/chloroform, gradient]	-	5,6-epoxycholesterol, 5,6-epoxysitosterol 5,6-epoxystigmasterol5,6;22,23-diepoxystigmasterol, 5,6;22,23-diepoxybrassicasterol	Giuffrida et al., 2004
HPLC-MS (APCI, iontrap)	C18 (150x4.0 mm, 5 µm) [methanol/acetonitrile/water 87:6:7 v/v, isocratic]	Pork, beef, chicken, egg	Cholesterol, 25-OH-cholesterol, 5α,6α-epoxycholesterol, 7-ketocholesterol, 7β-OH-cholestrol, cholesterol triol	Raith et al., 2005
HPLC-UV (210 nm)-RI HPLC-MS (APCI, linear ion trap)	CN column (300x3.9 mm, 4 µm) [hexane/isopropanol 96:4 v/v, 90:10 v/v, isocratic]	Egg	Cholesterol, 19-OH-cholesterol, 20α-OH-cholesterol, 22-OH-cholesterol, 24-OH-cholestreol, 25-OH-cholesterol, 5α,6α-epoxy-cholesterol, 5β,6β-epoxy-cholesterol,7-ketocholesterol, 7α-OH-cholesterol, 7β-OH-cholesterol, cholesterol-triol	Mazalli et al., 2006

2.4.2.2 Specific analysis of sterol hydroperoxides

Hydroperoxides can be determined with iodometric and Fe (III) complex methods, enzymatic methods and UV detection of conjugated diene hydroperoxides. While these methods can be used to determine the total amount of hydroperoxides, they lack specificity (Dobarganes et al., 2002). To obtain more information than the total amount of hydroperoxides, chromatographic methods, such as thin-layer chromatography (TLC), GC and LC, are needed (Hartvigsen et al., 2000). Sterol hydroperoxides have been mainly isolated and separated with TLC methods with specific colour reactions (Smith and Hill, 1972; Kulig and Smith, 1973). Visualization of hydroperoxide was accomplished by spraying the TLC plate with 1% N,N-dimethyl-p-phenylene-diamine dihydrochloride (Yanishlieva and Marinova, 1980), and other products are normally visualized after spraying with 50% sulphuric acid (Yanishlieva and Marinova, 1980; Blekes and Boskou, 1989). The hydroperoxides were reduced to corresponding hydroxides before final identification with GC-MS (Bortolomeazzi et al., 1999) or hydrogen nuclear magnetic spectroscopy (NMR) (Beckwith, 1989). TLC is a rapid method, but it exposes samples to potential oxidation as a result of its large surface area and also suffers from low loading capacity. A few studies on analysis of cholesterol hydroperoxides using HPLC with different detection methods have been published (Brown et al., 1997; Caboni et al., 1997; Geiger et al., 1997; Adachi et al., 1998; Chien et al., 1998; Yamazaki et al., 1999; Adachi et al., 2000).

Recently, new HPLC-CL (Adachi et al., 1998; Yamazaki et al., 1999) and HPLC-EC (Geiger et al., 1997) methods have been developed for the determination of lipid hydroperoxides. In addition to quantitative data, these methods provide information on the molecular structures of the compounds. One interesting approach to increase the sensitivity and specificity of HPLC analysis of hydroperoxides in NP chromatography systems has been the use of post-column reaction. Akasaka et al. (1988) and Ohshima et al. (1996) developed methods for specific detection of hydroperoxides using LC with fluorescence detection (FL) after post-column reaction with diphenyl-1-pyrenylphosphine (DPPP). The reagent has no fluorescence itself; instead, reaction of DPPP specifically with hydroperoxides leads to formation of the DPPP oxide, which has strong fluorescence (Figure 7) (Akasaka et al. 1987a; 1987b). The reaction of hydroperoxide with DPPP is quantitative, and therefore, the fluorescence intensity of the DPPP oxide directly shows the amounts of hydroperoxides (Akasaka et al., 1992). Unoxidized lipids, such as triacylglycerols, cholesterol, cholesterol esters, hydroxyl lipids and antioxidants, such as butylated hydroxytoluene (BHT), do not give response with this detection method (Akasaka and Ohrui, 2000). This method can be used with many solvents in isocratic or gradient mode, therefore being suitable for both NP and RP LC separation

(Akasaka et al, 1999; Akasaka and Ohrui, 2000). The UV detector can also be connected to the instrument before the derivatization process to get more information about the sample. The HPLC-UV-FL method is sensitive, simple and selective for detection of hydroperoxides, but final identification must always be made with other methods such as GC-MS. The HPLC-UV-FL method has been used for analysis of hydroperoxides at picomole levels from free fatty acids, triacylglycerol and cholesterol esters (Akasaka et al., 1999), methyl linoleate, methyl oleate and methyl linolenate (Ohshima et al., 1996) and phosphatidylcholine (Akasaka et al., 1988; Akasaka et al., 1999). To our knowledge, free cholesterol or plant sterol hydroperoxides have not been previously analysed with HPLC-UV-FL with post-column reagent addition.

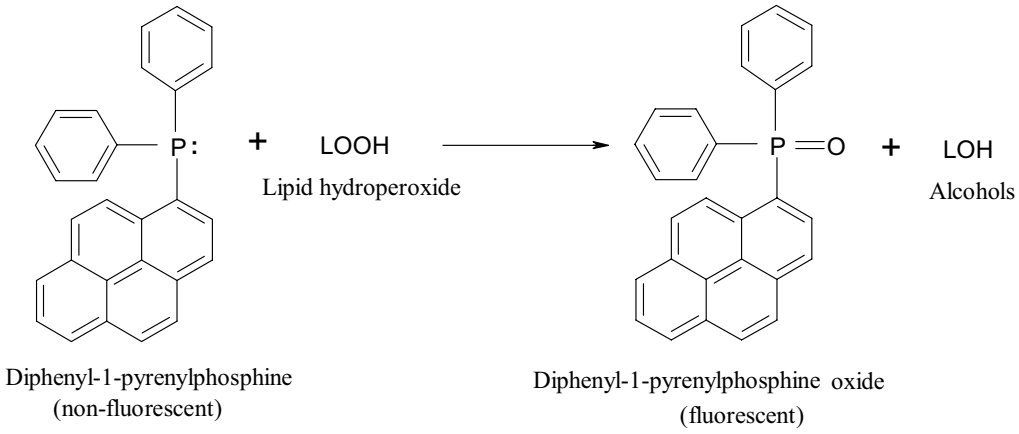

Figure 7. Reaction of DPPP with hydroperoxides (Akasaka and Ohrui, 2000).

2.4.2.3 Analysis of sterol oxides with LC-MS

The main problem present in LC methods is requirement of separation of different sterol oxides formed. Generally, foods and sterol ingredients contain a mixture of plant sterols, and oxidation thus leads to a complex mixture of oxidation products. When LC is coupled to MS, identification and quantification of totally or partly co-eluting compounds are possible. However, good separation between the oxides of the same parent sterol is needed because some oxides have the same molecular weight (Raith et al., 2005). Recently, a few reports of the analysis of cholesterol oxides by HPLC-MS have been published (Table 3) (Careri et al., 1998; Manini et al., 1998; Razzazi-Fazeli et al., 2000, Burkard et al., 2004; Raith et al., 2005).

Careri et al. (1998) used particle beam (PB) ionization for analysing cholesterol and some cholesterol oxides with LC-MS. Recently, LC-MS with atmospheric pressure chemical ionization (APCI) was found to be especially suitable for cholesterol oxide analysis. APCI instruments are commonly available. The APCI is based on chemical ionization carried out in an ion source operating at atmospheric pressure. Manini et al. (1998) were the first to evaluate the cholesterol oxide analysis with APCI LC-MS. They also applied the method for analysis of cholesterol oxides in beef and compared these results with those obtained from LC-UV and LC-MS with PB. Better sensitivity and selectivity were obtained using LC-MS with APCI ionization than by using the other methods. Razzazi-Fazeli et al. (2000) described development and application of an online APCI LC-MS interface for detection of seven cholesterol oxides in different foodstuffs. Burkard et al. (2004) developed RP HPLC-MS for quantification of two side-chain oxidation products in human plasma. Raith et al. (2005) developed RP LC-MS with APCI for quantification of cholesterol and five of its oxidation products in foods. To our knowledge, only Giuffrida et al. (2004) have used LC-MS for plant sterol oxide analysis. They studied epoxidation of unsaturated pure triacylglycerol, cholesterol and plant sterols. They analysed products with triple quadrupole mass spectrometry (MS) and ion-trap MS by using positive electrospray ionization (ESI). They reported for the first time formation of diepoxy compounds of stigmasterol and brassicasterol.

For cholesterol oxides, 7-ketocholesterol has been reported to have the protonated molecular $[M+H]^+$ as a base peak (Manini et al., 1998; Razzazi-Fazeli et al., 2000). For other cholesterol oxides, fragments representing the loss of one to three water molecules ($[M-H_2O+H]^+$, $[M-2H_2O+H]^+$ and $[M-3H_2O+H]^+$), depending on the number of oxygenated groups, were observed (Manini et al., 1998; Razzazi-Fazeli et al., 2000; Raith et al., 2005). Manini et al. (1998) and Razzazi-Fazeli et al. (2000) also observed mobile phase adducts. For hydroperoxides of triacylglycerols, fragments such as $[M-H_2O+H]^+$ and $[M-H_2O_2+H]^+$ were observed (Kusaka et al, 1996). Giuffrida et al. (2004) used ESI and ammonium formate to enhance ionization efficiency, obtaining fragments such as $[M+NH_4]^+$ in addition to $[M+H]^+$, $[M-H_2O+H]^+$ and $[M-2H_2O+H]^+$.

3. AIMS OF THE STUDY

When foods are enriched, the stability of the added component must be evaluated. In the case of plant sterols, oxidation is the reaction of main concern. To be able to control and prevent harmful oxidation, the oxidation mechanism and formation of individual oxidation products need to be elucidated. The aim of this research was to develop HPLC methods to identify and quantify both the primary and the secondary oxidation products of plant sterols. The methods must allow both formation and decomposition of thermolabile hydroperoxides and secondary oxidation products to be monitored, thus enabling investigation of the oxidation mechanism of plant sterols. Photo- and thermo-oxidized stigmasterol and thermo-oxidized plant sterol mixture were used as model samples. Stigmasterol was chosen as the model compound because it is commercially available in pure state.

To achieve the overall aim, the following more detailed objectives were set:

1. To develop an HPLC-UV-FL method for specific determination of plant sterol hydroperoxides and secondary oxidation products in simple sterol models (**I, II**).

2. To develop a LC-MS method for determination of plant sterol oxidation products in complicated sterol mixtures (**III, IV**).

3. To apply the HPLC methods to study the profiles of plant sterol oxides formed at different temperatures and under photo-oxidation (**I-IV**).

4. MATERIALS AND METHODS

This section summarizes the materials and methods presented in more detail in the original papers (**I-IV).**

4.1 Standards and model compounds (I-IV)

Stigmasterol (95%) from Sigma Chemical Co. (St. Louis, MO, USA) (**I, II**) and from Fluka Chemie AG (Buchs, Switzerland) (**III**) and β-sitosterol (**IV**) (containing 55% β-sitosterol, 26% campesterol, 13% stigmasterol and 6% brassicasterol) purchased from Sigma Chemical Co. were used as model compounds in oxidation experiments. The stigmasterol model sample was purified before the oxidation mechanism study (**II**). In brief, hydroperoxides were first reduced with sodiumborohydride ($NaBH_4$). The reaction was then quenched with water and excess reagent was destroyed with aqueous hydrochloric acid (HCl). Saturated aqueous sodium chloride (NaCl) was added and the solution was extracted with methyl-tert-butylether (MTBE). The organic layers were washed with water, dried with sodium sulphate (Na_2SO_4), filtered and evaporated to dryness. The residue was dissolved and purified by semipreparative NP HPLC (250 mm x 10 mm i.d., 5 µm; Supelco, Bellefonte, PA, USA) using MTBE:heptane (30:70) as a mobile phase at a flow rate of 3 ml/min. The concentration of stigmasterol was confirmed by using GC-FID (Lampi et al., 2002).

Plant sterol secondary oxidation products were quantified by using 5α,6α-epoxycholesterol (**III**, **IV**) and 7-ketocholesterol (**II-IV**) from Sigma Chemical Co. and 7α-OH-cholesterol (**II**) and 7β-OH-cholesterol (**III**, **IV**) from Steraloids (Wilton, NH, USA). The functioning of the HPLC-UV-FL and HPLC-MS instruments was checked with a methyl linoleate hydroperoxide solution (MeLo-OOH) (**I**, **II)**, and reserpine (**III**, **IV**), respectively.

19-OH-cholesterol purchased from Sigma Chemical Co. was used as an internal standard in the GC-FID and GC-MS analyses (**I-IV**). Performance of the GC-instruments was evaluated daily using the test solution containing the same concentrations of cholesterol, stigmasterol and dihydrocholesterol (Sigma Chemical Co.).

4.2 Photo-oxidation of stigmasterol (I)

Stigmasterol hydroperoxides formed during photo-oxidation were used for development of the hydroperoxide-specific HPLC-UV-FL method. Stigmasterol (20 mg) was dissolved in dichloromethane and photo-oxidized by using methylene blue as a sensitizer. Oxidation was

carried out in glass vials (45 mm x 25 mm i.d.) by using a 75-watt light source through a water layer. The time of photo-oxidation varied from 0 to 90 min, and the experiment was repeated twice. After photo-oxidation, dichloromethane was evaporated and the sample was dissolved in 4 ml of n-heptane:isopropanol (97:3, v/v) and filtered to remove the photosensitizer. Cholesterol was photo-oxidized with the same method to help in the final identification of the stigmasterol hydroperoxides.

4.3 Thermo-oxidation of plant sterols (II-IV)

Thermo-oxidized stigmasterol (**II-III**) and a sterol mixture (**IV**) were used to evaluate the suitability of the HPLC-UV-FL and LC-MS methods to study formation of plant sterol oxides. Stigmasterol (10 mg) (**II**, **III**) and a sterol mixture (10 mg) (**IV**) were heated in a thermostated oven at 100°C (**II**) and 180°C (**II-IV**). The heating time varied from 0 to 7 days at 100°C (**II**) and from 0 to 60 min at 180°C. The heating was done in open glass vials (22 mm x 46 mm). After oxidation, the samples were cooled in a dessicator and dissolved in 2 ml (**II, III**) or 5 ml (**IV**) of n-heptane:isopropanol (98:2, v/v) and filtered before HPLC analysis. The heating experiments were carried out in triplicate (**III-IV**) or six times (**II**), and each sample was analysed in duplicate.

4.4 Analysis of oxidation products (I-IV)

4.4.1 HPLC-UV-FL (I, II)

The hydroperoxide-specific HPLC-UV-FL method was developed to study oxidation reactions of sterols. The method was based on previously published post-column lipid hydroperoxide detection methods for other lipids (Akasaka et al., 1993b; Ohshima et al., 1996). The compositions of the mobile phase (**I-II**), elution programme and post-column conditions, including reagent concentration and reaction coil length (**II**), were modified based on preliminary experiments done with MeLo-OOH, cholesterol oxides and oxidized stigmasterol samples. The HPLC conditions were optimized by using secondary oxidation products of cholesterol.

The mobile phase and reagent pumps were Waters 510 (Milford, USA), the UV detector used as a general detector was Waters 486. The fluorescence intensity of the DPPP oxide was detected at 380 nm, after excitation at 352 nm, with a Hewlett Packard 1046 A (Waldbronn, Germany) FL detector.

The separation of stigmasterol oxides was performed by NP HPLC using an isocratic elution system with n-heptane-isopropanol (97:3, v/v) (**I**) or a linear gradient programme with isopropanol (from 2%-5%) and heptane (**II**) and a silica Supercosil column (250 mm x 2.1 mm i.d., 5 µm; Supelco, Bellefonte, PA, USA). The flow rate of the mobile phase was 0.6 ml/min. The HPLC eluent was monitored with the UV detector at 206 nm, prior to the post-column reaction with DPPP (Figure 8). The DPPP solution (0.75 mg DPPP/100 ml of 1-butanol-MeOH, 1:1 v/v, containing 25 mg BHT) was added to the eluent at a rate of 0.4 ml/min. The DPPP reagent reacted with hydroperoxides in a stainless steel coil (10 m x 0.5 mm i.d.) (**I**), (20 m x 0.5 mm i.d.) (**II**) at 80°C. After reaction, the eluent from the coil was cooled to room temperature by passage through a short stainless steel coil (0.5 m x 0.5 mm i.d.) placed in a water jacket. The injection volume was 50 µl and all samples were injected twice. The specificity of the detection was investigated with secondary oxidation products of cholesterol (**I**) because plant sterol oxides were not commercially available. Functioning of the instrument was checked daily with MeLo-OOH by monitoring the retention time and detector response. Data handling was performed using Millenium 2010 software (Waters, Milford, USA).

To confirm the structures of stigmasterol hydroperoxides formed, cholesterol hydroperoxides were produced with the same photo-oxidation method used for stigmasterol. After fractionating and reduction of hydroperoxides (**I-II**) and fractionating of secondary oxidation products (**II**), oxidation products were identified with GC-MS by comparing mass spectral data and retention times with those of the corresponding hydroxy derivatives of cholesterol. A sterol hydroperoxide standard was not commercially available; hence, the amounts of stigmasterol hydroperoxides were estimated with peroxide value (PV) measurements (**I**) or quantified by using MeLo-OOH solution as an external standard (**II**). The concentration of MeLo-OOH was measured by a UV detector at a wavelength of 234 nm (Hopia et al., 1966). The secondary oxidation products of stigmasterol were quantified by using 7-ketocholesterol and 7α-OH-cholesterol (**II**). 6α-OH-3-ketostigmasterol, 6β-OH-3-ketostigmasterol and 7-ketostigmasterol were quantified by using 7-ketocholesterol, and 5α,6α-epoxystigmasterol, 5β,6β-epoxystigmasterol, 6β-OH-stigmasterol, 7β-OH-stigmasterol and 7α-OH-stigmasterol by using 7α-OH-cholesterol. All standards (MeLo-OOH, 7-ketocholesterol and 7α-OH-cholesterol) were in the same standard solution. Five concentration levels were used, with a range of 3.1-92.3 µg/ml for MeLo-OOH, 2.0–485.9 µg/ml for 7-ketocholesterol and 2.0–499.2 µg/ml for 7α-OH-cholesterol. Calibration curves were analysed at the beginning of each of the six oxidation experiments. The detection limits were calculated based on a signal-to-noise ratio of 3.

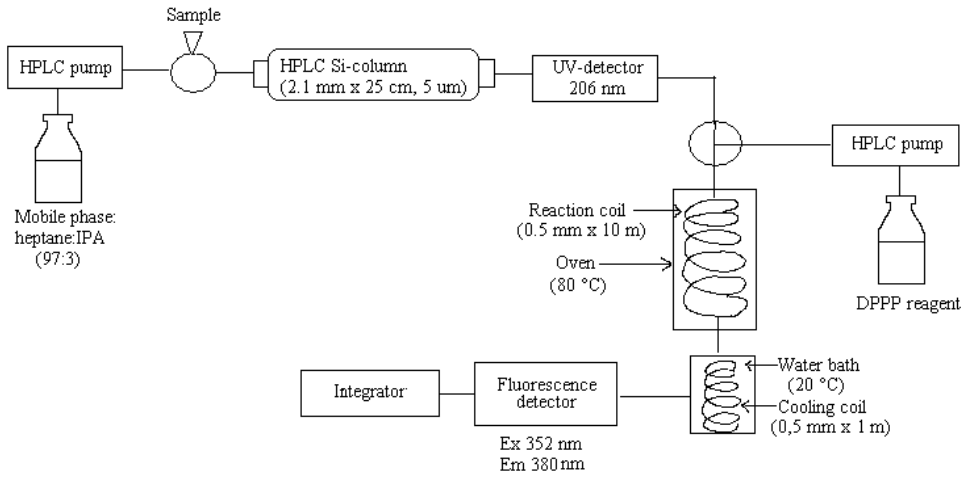

Figure 8. The HPLC-UV-FL instrument (**I**).

4.4.2 LC-MS (III, IV)

The LC-MS method was developed for sensitive detection of hydroperoxides and secondary oxidation products, including epoxides and triol. Applicability of the method was investigated using thermo-oxidized stigmasterol (**I**) or sterol mixture (**II**) as model samples. The LC instrument used was Agilent 1100, and direct online MS detection was carried out with a quadrupole ion-trap (QIT) mass spectrometer (Esquire-LC, Bruker Daltonik, Bremen, Germany) using an APCI positive ion scanning mode.

The LC gradient was optimized by using nine commercially available cholesterol oxidation products. The MS parameters were optimized using 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-OH-cholesterol. The optimized parameters are presented in Table 4.

Table 4. Optimized MS parameters.

MS parameter	Time segment		
	5α,6α-epoxycholesterol	7-ketocholesterol	7β-OH-cholesterol
	0-9.5 min (III) 0-10 min (IV)	9.5–15.5 min (III) 10–14 min (IV)	15.5–40 min (III) 14–40 min (IV)
Dry gas temperature	300°C	300°C	300°C
Flow rate	7.0 l/min	7.0 l/min	7.0 l/min
APCI interface temperature	300°C	300°C	300°C
Nebulizer gas pressure	206.9 kPa	206.9 kPa	206.9 kPa
Corona discharge needle	3500 V	3500 V	3500 V
Capillary	-3600 V	-3600 V	-3600 V
End-plate offset	-750 V	-750 V	-750 V
Skimmer I	27.5 V	27.5 V	27.5 V
Skimmer II	6.5 V	6.5 V	6.5 V
Octopole	2.7 V	2.7 V	2.7 V
Octopole Δ	2.3	2.3	2.3
Octopole RF	160.7	160.7	160.7
Capillary exit offset	38 V	85 V	38 V
Capillary exit	65.5 V	112.5 V	65.5 V
Trap drive	44	44	44.6

Separation was performed on a silica Supercosil column (250 mm x 2.1 mm i.d., 5 μm; Supelco, Bellefonte, PA, USA) with gradient of heptane (solvent A) and isopropanol (solvent B) programmed as follows: 0-15 min 2% B, 15-35 min from 2% B to 20% B, 35-40 min from 20% B to 2% B and post-run 30 min with 2% B. The total flow rate of the mobile phase was 0.6 ml/min. The trap ion current control value was set at 35 000 units, and the maximal accumulation time was 200 ms. The APCI mass spectra were recorded using scan range 150-700 m/z and summation of eight spectra. Collision-induced dissociation (CID) was produced with helium 0.6 mPa (**III-IV**). The performance of the APCI was checked before experiments by using reserpine. The injection volume of plant sterol samples was 1 μl (**III**) or 3 μl (**IV**), and in the case of cholesterol standards 1 μl (**III-IV**). When fragmentation of plant sterols was studied, an injection volume of 6 μl was used (**IV**).

Quantitative determinations of sitosterol (**IV**), campesterol (**IV**), stigmasterol (**III-IV**) and brassicasterol (**IV**) oxidation products were made using 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-OH-cholesterol as external standards. 6α-OH-3-keto-, 6β-OH-3-keto-, 6-keto- (**IV**) and 7-ketositosterol (**IV**), campesterol (**IV**), stigmasterol (**III**, **IV**) and brassicasterol (**IV**) were quantified by using 7-ketocholesterol and 5α,6α-epoxy- and 5β,6β-

epoxysitosterol (**IV**), campesterol (**IV**), stigmasterol (**III**, **IV**) and brassicasterol (**IV**) with 5α,6α-epoxycholesterol. Similarly, 6β-OH-, 7β-OH- and 7α-OH of sitosterol (**IV),** campesterol (**IV**), stigmasterol (**III**, **IV**) and brassicasterol (**IV**) were quantified by using 7β-OH-cholesterol. All compounds were quantified by using extracted ion chromatograms obtained by the sum of the main m/z values representing fragments $[M+H]^+$, $[M–H_2O+H]^+$and $[M–2H_2O+H]^+$. The concentrations of calibration solutions were measured with GC-FID (Lampi et al., 2002). Five concentration levels were used with ranges of 7.9-370.3 μg/ml (**III**) or 4.2-197.8 μg/ml (**IV)** for 5α,6α-epoxycholesterol, 8.6-404.7 μg/ml (**III**) or 4.7-219.2 μg/ml (**IV**) for 7-ketocholesterol and 11.0-525.1 μg/ml (**III**) or 6.0-279.3 μg/ml (**IV**) for 7β-OH-cholesterol. The calibration curve was measured before (**III**, **IV**) and after (**IV**) each oxidation experiment. Calculation of the detection limits for the compounds studied was based on a signal-to-noise ratio of 3. All samples were analysed twice.

4.4.3 GC-FID and GC-MS (I-IV)

GC-FID was used for measuring the concentrations of the cholesterol standard solutions (Lampi et al., 2002) and GC-MS for plant sterol oxide structure confirmation (**I-IV**) (Soupas et al., 2004a; Soupas et al., 2005). The reliability of the quantification of oxides from a complicated sterol mixture with LC-MS, was confirmed by analysing same samples with GC-FID and GC-MS (**IV**). In brief, for structure confirmation, all oxidation products, including hydroperoxides and secondary oxidation products, were first fractionated by using HPLC-UV with an analytical silica column. The hydroperoxide fractions were reduced to corresponding hydroxides before GC-MS analysis, as presented below. The hydroperoxide fractions were evaporated, dissolved in diethylether/methanol (1:1) and reduced with an excess of $NaBH_4$ (Bortolomeazzi et al., 1999). The reduction was quenched with water. The excess reduction reagent was destroyed with HCl, after which saturated NaCl was added and the solution was extracted with diethyl ether. The organic layers were washed with water, dried with Na_2SO_4 and filtered. The residue was evaporated and dissolved in n-heptane-isopropanol (97:3, v/v). To ensure that all hydroperoxides were completely reduced, the solution was analysed with the HPLC-UV-FL method. After fractionating and reduction of hydroperoxides, an adjusted amount of the internal standard (19-OH-cholesterol) was added to the samples. The samples were evaporated, dissolved and purified with SiOH-solid phase extraction (SPE) if they contained unoxidized sterols. All samples were then subjected to overnight silylation by a bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1) mixture. The reagent was evaporated and TMS ether derivatives were dissolved in a fixed volume of heptane.

In the GC-FID method, quantification was performed by using a general relative response factor of 1.00 (Lampi et al., 2002), and in the GC-MS method, indirectly via GC-FID, as described by Soupas et al. (2004a; 2005), using one target and one qualifier ion for each oxidation product. The selective ion monitoring (SIM) mode was used for quantification. A sterol standard mixture was used for daily evaluation of the GC instruments.

4.5 Statistical analysis (I, III, IV)

Linearity of the calibration graphs was evaluated using regression analysis, and comparison of regression lines was performed with Statgraphics Plus 4.0 software (Manugistics Inc., Rockville, MD, USA) (**III-IV)**. Statgraphics Plus 4.0 paired-sample comparison was used to compare the results from LC-MS with those from GC-FID and GC-MS (**IV**). HPLC-UV-FL results and PV values were compared by linear regression analysis using Statgraphics 3.0 software (Manugistics) (**I**).

5.RESULTS

This section summarizes the results presented in studies **I-IV**.

5.1 HPLC separation (I-IV)

NP chromatography separated positional and epimeric isomers of sterol oxides. With isocratic elution (heptane:isopropanol 97:3 (v/v)), 5α-OOH-, 6α,β-OOH- and 7α-OOH-stigmasterol formed during photo-oxidation were separated from each other (**I**; Figure 3A and 3B). The peak eluting together with the unoxidized stigmasterol with a retention time of 4 min (RT 4) could not be identified. The secondary oxidation products were not detected because oxidation had not proceeded sufficiently far. In the case of thermo-oxidized stigmasterol, the best resolution was achieved by using a linear gradient of isopropanol (from 2% to 5%) and heptane (**II**), and under these conditions seven primary and eight secondary oxidation products were separated (**II**; Figure 1). In both cases, 6α-OOH- and 6β-OOH-stigmasterol coeluted (**I,II**). When stigmasterol and cholesterol oxides (**III**) and mixtures of plant sterol oxides (**IV**) were analysed, the gradient elution with heptane (A) and isopropanol (B) was used. The separation conditions were optimized with commercially available cholesterol oxides (Figure 9), and the best elution programme was as follows: 0-15 min 2% B, 15-35 min from 2% to 20% B, 35-40 min from 20% to 2% B and post run 30 min with 2% B. With this gradient elution, nine plant sterol secondary oxidation products (**III**; Figure 2 and **IV**; Figure 1A-C) and two hydroperoxides (**III**; Figure 2), which eluted in the same order as the corresponding cholesterol oxidation products, could be separated. On the other hand, sitosterol, campesterol, stigmasterol and brassicasterol oxides with the same functionalities coeluted. However, they differ in their molecular weights, and identification and quantification were thus possible with LC-MS. By connecting LC with MS, as many as 34 plant sterol oxides were able to be determined with a single LC run (**IV**).

The NP methods used were rapid; separation of hydroperoxides was achieved in 15 min (**I**) and separation of plant sterol secondary oxidation products within 21 min (**II**) and 23 min (**III, IV**). Resolution between oxides detected by both UV and FL detectors was good (**I, II**), except for that of 6α-OOH-stigmasterol and 6β-OOH-stigmasterol. Chromatographic separation was good with LC-MS, and the resolution between the critical pair of 7β-OH-cholesterol and 7α-OH-cholesterol was 1.3 (**III**). Retention times were reproducible between chromatographic runs (**I-IV**).

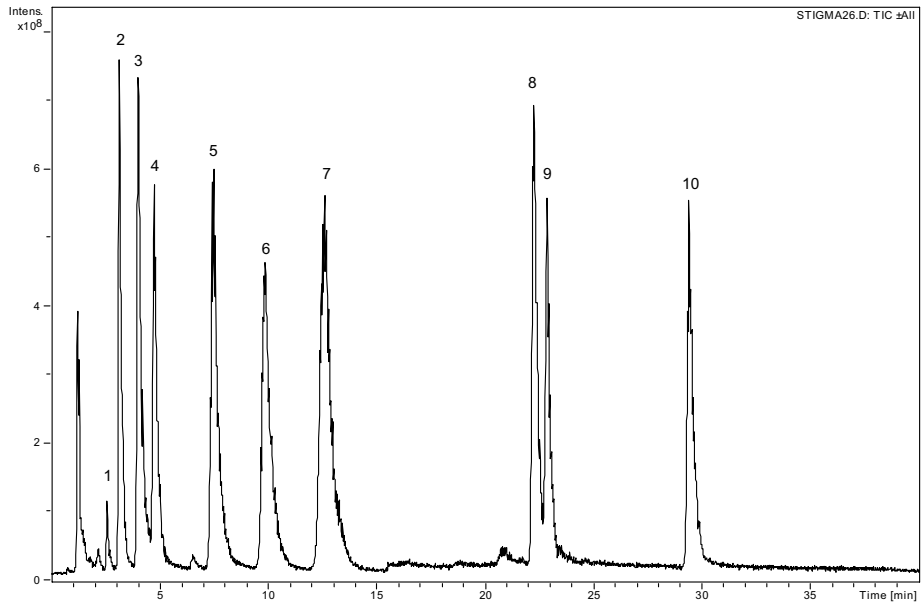

Figure 9. Separation of cholesterol oxide standards using the LC-MS method. 1 = cholesterol, 2 = 20α-OH-cholesterol, 3 = 22-OH-cholesterol, 4 = 25-OH-cholesterol, 5 = 5α,6α-epoxycholesterol, 6 = 6-ketocholesterol, 7 = 7-ketocholesterol, 8 = 7β-OH-cholesterol, 9 = 7α-OH-cholesterol, 10 = cholesterol-triol.

5.2 HPLC-UV-FL (I, II)

The HPLC-UV-FL method allowed monitoring of the formation and decomposition of both primary and secondary oxidation products, and thus, the overall picture of the oxidation status was achieved with a single HPLC analysis, as shown in Figure 10. With the UV detector, an unoxidized sterol and all oxidation products could be detected, and with the FL detector hydroperoxides formed could be detected specifically.

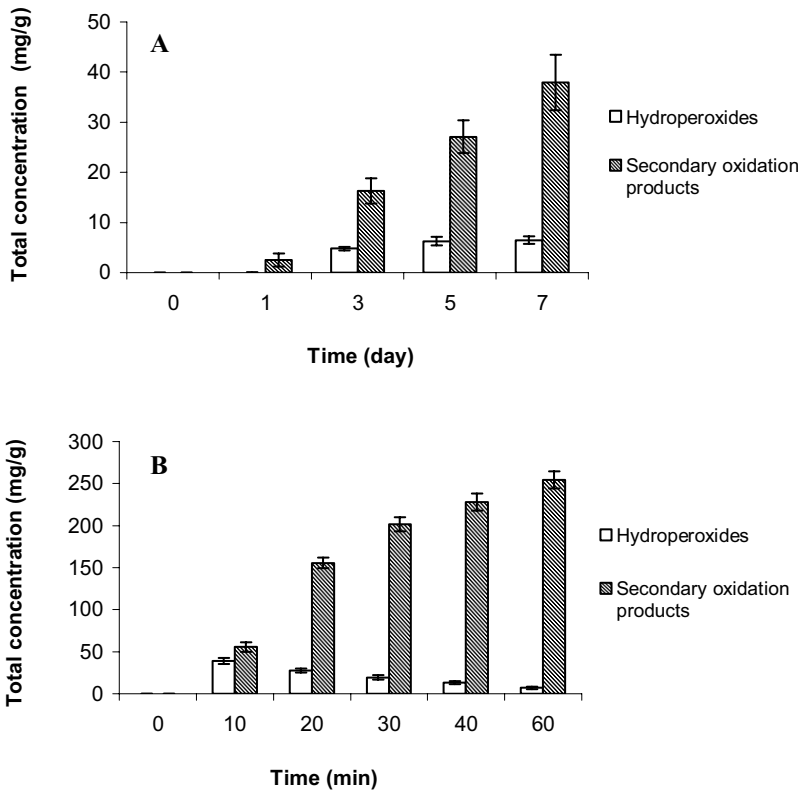

Figure 10. Total hydroperoxides and secondary oxidation products (mg/g of sterol) after thermo-oxidation at 100°C (**A**) and 180°C (**B**) analysed by the HPLC-UV-FL method (**II**).

The specificity of the HPLC-UV-FL method was studied with commercially available 25-OH-cholesterol, 5α,6α-epoxycholesterol, 7-ketocholesterol, 7α-OH-cholesterol and cholesterol-triol at concentration levels of 0.6-1.4 mg/ml (**I**). The results showed that secondary oxidation products of cholesterol were unable to oxidize DPPP to fluorescent DPPP oxide. When the effect of coil length was evaluated, the results revealed that with longer coils larger peak areas were obtained. However, analysis time was longer and peak broadening became a problem (**II**). A coil length of 20 m was therefore deemed optimal. Higher DPPP concentrations increased background noise.

Stigmasterol hydroperoxides formed during photo-oxidation (**I**) and thermo-oxidation (100°C and 180°C) (**II**) were identified after being fractionated and reduced by GC-MS. Fractionated

secondary oxidation products were also identified with GC-MS (**II**). Retention times and mass spectral ions of TMS ethers and their relative ion abundances are presented in study **I** (**I**; Tables 1 and 2) and study **II (II**; Table 1). Identification of the major hydroperoxides formed during photo-oxidation; 6α,β-OOH-, 5α-OOH- and 7α-OOH-stigmasterol, and four of the seven hydroperoxide products formed during thermo-oxidation; 25-OOH-, 6α,β-OOH-, 7α-OOH- and 7β-OOH-stigmasterol, was successful. The compounds eluted at retention times of 4 min (RT 4) (**I**), 6 min (RT 6) and 7 min (RT 7) (**II**) together with unoxidized stigmasterol, and at the retention time of 9 min (RT 9) (**II**) together with 6β-OH-3-ketostigmasterol could not be fractionated and further identified. The RT4 compound was less polar than the other hydroperoxides formed during photo-oxidation and had a UV absorption maximum at 234 nm (**I**). Eight secondary oxidation products, 6β-OH-3-keto-, 6α-OH-3-keto-, 5α,6α-epoxy-, 5β,6β-epoxy-, 7-keto-, 6β-OH-, 7β-OH- and 7α-OH-stigmasterol, were also identified (**II).** Identification of 6α-OH-3-ketostigmasterol and 6β-OH-3-ketostigmasterol was tentatively done based on their retention times and fragmentation behaviour.

Secondary oxidation products were quantified with 7-ketocholesterol and 7α-OH-cholesterol. MeLo-OOH was used for quantification of hydroperoxides. Responses of 7-ketocholesterol and 7α-OH-cholesterol standards were linear with coefficients of determinations (R^2) over 0.999, and for MeLo-OOH, 0.997, respectively. The detection limit for 7α-OH-cholesterol, 7-ketocholesterol and MeLo-OOH were 19.4 ng/injection, 5.3 ng/injection and 19.2 ng/injection, respectively. Coefficient of variation (CV) values for all oxidation products were typically below 20%.

The FL detector responses of the HPLC-UV-FL method were compared with PVs (**I**). The results showed that the PV measurements and the FL detector were correlated (Figure 11). A significant correlation between FL results and PVs was observed at the 99% ($p<0.01$) confidence level. The correlation coefficient (r) obtained was 0.997.

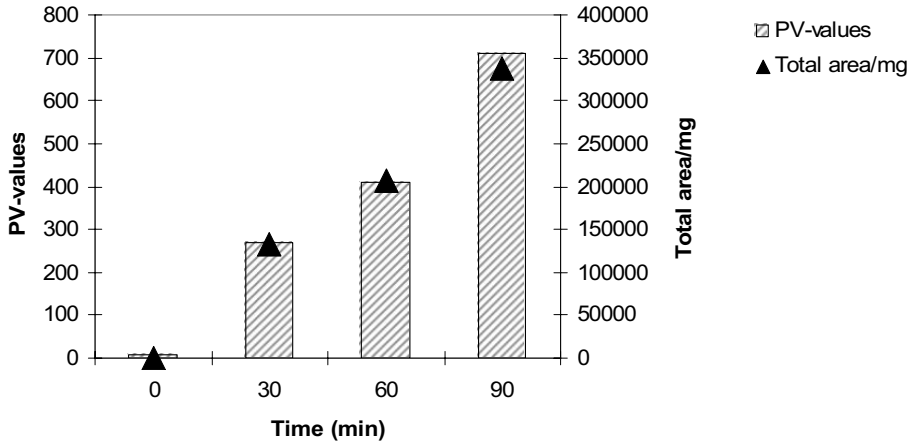

Figure 11. PV-values and total peak areas of stigmasterol hydroperoxides analysed by HPLC-UV-FL during photo-oxidation.

5.3 LC-MS (III, IV)

The LC-MS method was shown to be sensitive for detection of all main oxidation products formed during oxidation of sterols and sterol mixtures (**III-IV**). The method was optimized with commercially available cholesterol oxidation products (**III**). The MS parameters were optimized by using 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-OH-cholesterol. Despite the optimization procedure, the in-source fragmentation of certain pseudomolecule ions could not be avoided. When the dry gas and APCI interface temperatures were optimized, the signal intensities of the two main ions $[M+H]^+$ and $[M-H_2O+H]^+$ were followed. The results showed that an optimum temperature for 5α,6α-epoxycholesterol and 7-ketocholesterol was 300°C, whereas for 7β-OH-cholesterol the best signal was recorded at 350°C. Therefore, the temperature of 300°C for dry gas and APCI interface was chosen because temperature could not be changed during the run. Over the temperature range studied, no changes in fragmentation patterns were observed. With this method, both hydroperoxides and secondary oxidation products could be analysed, including epoxy and triol-compounds, which are difficult to analyse with traditional HPLC methods.

Fragmentation behaviour and relative ion abundances of cholesterol (**III**; Table 1A), stigmasterol (**III**; Table 1B and **IV**; Table 1) and sitosterol, campesterol and brassicasterol oxides (**IV**; Table 1) are presented in the tables. 6α-OH-3-keto, 6β-OH-3-keto and 7-keto compounds had a protonated molecule ion $[M+H]^+$ as the base peak. The protonated molecule ion was also detectable in the spectra of 5α,6α-epoxysitosterol (**IV**), 5α,6α-epoxycholesterol

(**III**), 5α,6α-epoxystigmasterol, 5β,6β-epoxystigmasterol (**III**), 6-ketocholesterol (**III**), 6-ketositosterol and 6-ketocampesterol (**IV**) spectra, althought with low abundances. In general, fragments indicating a loss of one to three water molecules, $[M\text{-}H_2O+H]^+$, $[M\text{-}2H_2O+H]^+$ and $[M\text{-}3H_2O+H]^+$, were observed. In addition, fragments representing loss of hydrogen peroxide $[M\text{-}H_2O_2+H]^+$ or hydrogen peroxide and water $[M\text{-}H_2O_2\text{-}H_2O+H]^+$ were present in mass spectra of 7α-OOH- and 7β-OOH-stigmasterol (**III**). The results showed that sitosterol, campesterol, stigmasterol and cholestrol oxides had similar fragmentation behaviour, only their relative ion abundances were slightly different (**III, IV**). The fragmentation pattern of brassicasterol oxides could not be achieved with tandem MS^2 and MS^3 because of inadequate amounts of brassicasterol in the sterol mixture (**IV**). With this method, the mass spectra of 20α-OH-cholesterol, 22-OH-cholesterol, several stigmasterol oxides (**III**) and sitosterol and campesterol oxides (**IV**), which have not been previously described, were obtained.

With the LC-MS method, 8 stigmasterol (**III**) and 34 plant sterol oxides (**IV**) formed during thermo-oxidation at 180°C were quantified. The quantification was carried out with cholesterol standards using an external standard method, and the extracted ion chromatogram of the sum of the main fragments of different oxidation products as presented in Figure 2 (**III**) and Figure 1A-C (**IV**). 7α-OOH- and 7β-OOH-stigmasterol were not quantified because sterol hydroperoxy standards were unavailable commercially. The linearity of the calibration graphs was excellent (**III,** Table 2; **IV**, Table 2). The detection limits ranged from 0.09 (**IV**) to 0.3 ng/injection (**III**) for 5α,6α-epoxycholesterol, from 0.10 (**IV**) to 0.3 ng/injection (**III**) for 7-ketocholesterol and from 0.13 (**IV**) to 0.3 ng/injection (**III**) for 7β-OH-cholesterol. The CV values of oxides formed were usually below 20%; only at very low concentrations were CV values over 20%. The quantified amounts of epoxides were higher than those previously obtained under the same oxidation conditions by the HPLC-UV-FL method.

5.4 Comparison of HPLC-UV-FL, LC-MS, GC-FID and GC-MS methods

The amounts of stigmasterol oxidation products formed at the last time-point during photo-oxidation and thermo-oxidation at 100°C and 180°C, analysed with different methods in the different studies, are listed in Table 5. With the HPLC-UV-FL method, the main oxidation products formed during thermo-oxidation or photo-oxidation could be analysed. The concentrations of all stigmasterol secondary oxidation products, except epoxides, were higher when analysed with HPLC-UV-FL than with LC-MS. The number of double bonds had an effect on the quantification results obtained with the UV detector. Following the oxidation was possible with the HPLC-UV-FL because hydroperoxides could be detected specifically with FL detection. Separation between oxides was required when using HPLC-UV-FL, and

analysis of oxides of only one plant sterol was possible. Coelution of oxides and also unoxidized sterols was a problem when using GC-FID. MS detector combined with the LC or GC instrument enabled the identification and quantification of partially and totally coeluted compounds. The advantage of LC-MS compared with GC-MS was, in addition to less sample pretreatment, its ability to analyse thermolabile primary oxidation products. Moreover, the sensitivity was 20-200 times better with LC-MS than with HPLC-UV-FL.

To elucidate the reliability of quantification with the LC-MS method, the thermo-oxidized plant sterol mixture was also analysed with GC-FID and GC-MS (**IV**; Tables 3, 4 and 5). The results showed that all methods gave similar formation patterns of sterol oxides during heating. The LC-MS gave quite similar results to GC-FID (**IV**; Figure 2A-C), although a sign test showed a difference at the 95% confidence level. The GC-MS results were higher, especially for 5β,6β-epoxy sterols. The difference between the results obtained was smallest in the case of 7β-OH-sitosterol.

Table 5. Amounts of stigmasterol products formed (mg/g) at the last time-point during photo-oxidation and thermo-oxidation. Products identified only are indicated with x.

Stigmasterol oxidation product	**Photo-oxidation 90 min**	**Thermo-oxidation 100°C 7 days**	**Thermo-oxidation 180°C 60 min**	
	HPLC-UV-FL (I)	**HPLC-UV-FL (II)**	**HPLC-UV-FL (II)**	**LC-MS (III)**
25-OOH		1.7 ± 0.1	0.8 ± 0.1	
5α-OOH	x			
6α-OOH	x	x	x	
6β-OOH	x	x	x	
7α-OOH	x	0.9 ± 0.0	0.8 ± 0.2	x
7β-OOH		1.3 ± 0.1	2.3 ± 0.1	x
6β-OH-3-keto		2.2 ± 1.5	33.4 ± 2.0	4.5 ± 0.5
6α-OH-3-keto		0.9 ± 0.1	17.3 ± 1.0	6.8 ± 0.6
5α,6α-epoxy		1.3 ± 0.5	7.0 ± 0.6	18.5 ± 0.8
5β,6β-epoxy		3.1 ± 1.1	14.2 ± 0.5	23.3 ± 0.9
6-keto				
7-keto		12.2 ± 1.4	67.0 ± 2.8	40.0 ± 15
6β-OH		2.8 ± 0.6	15.3 ± 1.3	5.5 ± 0.9
7β-OH		9.0 ± 1.1	55.6 ± 0.4	20.3 ± 1.4
7α-OH		6.4 ± 0.7	44.9 ± 1.6	14.0 ± 0.9
triol				x

5.5 Oxidation of plant sterols

5.5.1 Photo-oxidation (I)

When stigmasterol was photo-oxidized in the presence of methylene blue as a sensitizer, 5α-OOH-stigmasterol was formed as the main product together with smaller amounts of 6α-OOH-, 6β-OOH- and 7α-OOH-stigmasterol (**I**; Figure 4). Formation of 7β-OOH-stigmasterol was not observed. The amount of hydroperoxides increased throughout the study period (0-90 min). The amount of 7α-OOH-stigmasterol started to increase more sharply than the other products after 60 min of oxidation. Formation of secondary oxidation products was not seen since the oxidation had not proceeded sufficiently far.

5.5.2 Thermo-oxidation (II-IV)

Formation of plant sterol oxidation products during heating at 100°C (**II**) and at 180°C is presented in Figure 2 (**II**), Figure 3 (**III**) and Table 3 (**IV**). At 180°C, the amounts of hydroperoxides increased sharply during the first 10 min and then started to decrease (**II**). At 180°C, 7β-OOH-stigmasterol was the main product formed, and thus, the formation of hydroperoxides at position C-7 seemed to be more favourable than at position C-25. The formation and degradation of 7α-OOH-stigmasterol and 7β-OOH-stigmasterol were observed also when thermo-oxidized stigmasterol was analysed with LC-MS. At 180°C, the 7-keto compounds of plant sterols were the main secondary oxidation product formed after 60 min of oxidation (**II-IV**). All stigmasterol secondary oxidation products, except 7-ketostigmasterol, reached a plateau or started to decrease after 30 min (**III**) to 40 min (**II**) of oxidation. In the case of thermo-oxidized sterol mixture, all products formed, except 5β,6β-epoxy, 7β-OH and 7α-OH, increased throughout the study (**IV**). At 100°C, the amount of hydroperoxides and secondary oxidation products increased over the entire 7-day oxidation period (**II**). At 100°C, the main hydroperoxy formed was 25-OOH-stigmasterol and the main secondary oxidation product was 7-ketostigmasterol. At 100°C, the oxidation rate was much slower than at 180°C. The same oxidation products were formed at both temperatures, but the distribution of the products differed (**II-IV**). β-isomers of epoxy and hydroxy sterols were dominant throughout the experiments. Our results showed that all plant sterols with one double bond in the ring structure had a similar oxidation profile (**IV**), as shown in Figure 12. The double bond in the side-chain in stigmasterol and brassicasterol did not change the reactivity.

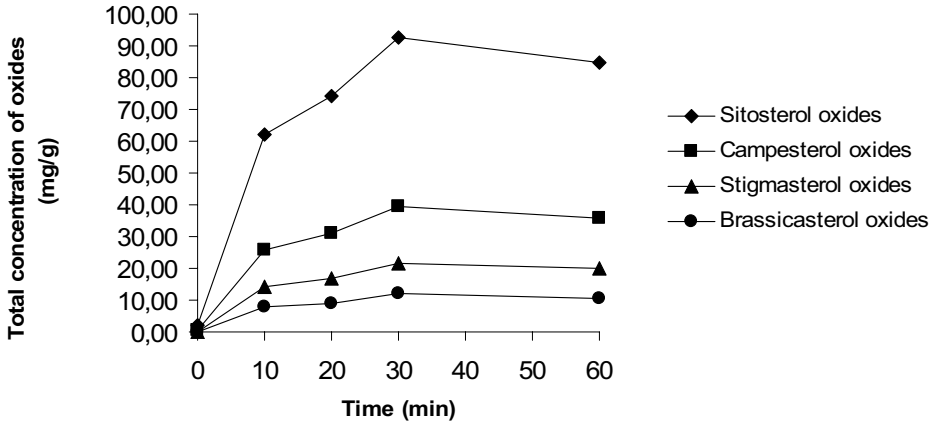

Figure 12. Total concentration of oxides of each sterol formed during the thermo-oxidation of the sterol mixture at 180°C over different time periods (**IV**).

6. DISCUSSION

6.1 Evaluation of HPLC methods

To control and prevent plant sterol oxidation, the oxidation mechanism and especially the formation and degradation of individual oxides must be elucidated. For this reason two HPLC methods for plant sterol oxide analysis were developed. With these HPLC-UV-FL and LC-MS methods, the formation and degradation of both thermolabile hydroperoxides and secondary oxidation products could be followed. This enables important information about the oxidation mechanism of sterols to be obtained. The applicability of the methods was evaluated by using photo-oxidized and thermo-oxidized stigmasterol and a thermo-oxidized sterol mixture as model samples. No sample pretreatment other than dissolving and filtration was needed since no matrix was present. So far, only one report of plant sterol oxide analysis by HPLC has been published (Giuffrida et al., 2004).

NP chromatography was used with both methods because of its ability to separate both positional and epimeric isomers. Isocratic conditions were used only in the first study, when stigmasterol hydroperoxides formed during photo-oxidation were analysed. In the rest of the studies, gradient elution was used to obtain better resolution over the wide polarity range of products formed during thermo-oxidation. Maerker et al. (1988) noted that cholesterol oxides with different polarities could not be separated with NP chromatography without gradient elution.

6.1.1 HPLC-UV-FL

With the HPLC-UV-FL method, the overall picture of the oxidation state of stigmasterol, which was used as a model sample, was achieved. Major oxidation products of sterols containing at least one double bond and also unoxidized sterol can be detected with UV at 206 nm, and hydroperoxides can be detected specifically with FL after post-column reagent DPPP addition. The HPLC-UV-FL method was more sensitive and selective than TLC methods used before for cholesterol and plant sterol hydroperoxides (Smith and Hill, 1972; Kulig and Smith, 1973; Yanishlieva and Marinova, 1980; Blekes and Boskou, 1989).

The coil length and DPPP reagent concentration of the post-column detection method were optimized. By using longer coils, larger peak areas were obtained, but peak broadening became a problem, similarly to the study of Akasaka et al. (1999) on free fatty acid, phosphatidylcholine, triacylglycerol and cholesterol ester hydroperoxides. The concentration of the reagent had an effect on the baseline, and with higher reagent concentrations the

background noise increased. When a linear gradient of isopropanol and heptane was used, no baseline drift was observed. Akasaka et al. (1999) did, however, note that irregular baseline drift sometimes occurs when undistilled isopropanol is used as eluent. Specificity of the method was confirmed by using commercially available secondary oxidation products of cholesterol. The results confirmed that these cholesterol oxidation products were unable to oxidize DPPP to the fluorescent DPPP oxide. The specificity of the method was also investigated by Akasaka et al. (1987b, 2000). They reported that DPPP reacts specifically with only hydroperoxides, while unoxidized lipids, hydroxyl lipids and antioxidants were unable to oxidize DPPP.

Final identification of the individual hydroperoxides and secondary oxidation products was achieved with GC-MS. The results revealed that TMS ethers of stigmasterol oxides have similar fragmentation patterns, as reported before by Bortolomeazzi et al. (1999) and Dutta (1997). Three hydroperoxides formed during thermo-oxidation (RT6, RT7 and RT9) and one hydroperoxide (RT4) formed during photo-oxidation were unable to be identified owing to coelution with unoxidized stigmasterol (RT4, RT6 and RT7) or 6β-OH-3-ketostigmasterol (RT9). Based on its retention time, the RT4 was less polar than the other hydroperoxides formed during photo-oxidation. Further, it had a UV absorption maximum at 234 nm, indicating that it contained a conjugated diene structure. 6β-OOH-3-ketostigmasterol and 6α-OOH-3-ketostigmasterol can be assumed to be among those unidentified hydroperoxides (RT6, RT7 and RT9) because both 6β-OH-3-ketostigmasterol and 6α-OH-3-ketostigmasterol were found among the secondary oxidation products. In addition, Yanishlieva et al. (1983) reported that when free sitosterol was oxidized at a temperature above 100°C, 6β-OOH-3-ketositosterol was formed, together with other oxidation products. It can also be assumed that unidentified hydroperoxides may include side-chain hydroperoxides such as 20-OOH- or 24-OOH-stigmasterol.

When the overall performance of the HPLC-UV-FL method was evaluated, the biggest challenge proved to be quantification. The plant sterol oxide standards were not commercially available. Therefore, MeLo-OOH was used for quantification of stigmasterol hydroperoxides and commercially available 7-ketocholesterol and 7α-OH-cholesterol for quantification of secondary oxidation products of stigmasterol. UV absorption is influenced by the number of double bonds. Therefore, if the standards used contained a different number of double bonds than the analyte quantified with it, the results may have been inaccurate. In this study, stigmasterol oxides had one double bond more than cholesterol oxides because stigmasterol has a double bond in the side-chain. Compounds with conjugated diene structure, i.e. 6α-OH-3-ketostigmasterol, 6β-OH-3-ketostigmasterol and 7-ketostigmasterol, were quantified by

using 7-ketocholesterol, and other products, i.e. 5α,6α-epoxystigmasterol, 5β,6β-epoxystigmasterol, 6β-OH-stigmasterol, 7β-OH-stigmasterol and 7α-OH-stigmasterol, were quantified with 7α-OH-cholesterol. 7-ketocholesterol has two double bonds in a conjugated diene position in the ring structure, whereas stigmasterol oxides quantified with it have two double bonds in conjugated diene position in their ring structure and one double bond in their side-chain. Products quantified with 7α-OH-cholesterol, which contained one double bond in the ring structure, had only one side-chain double bond (5α,6α-epoxystigmasterol and 5β,6β-epoxystigmasterol) or had one double bond in the ring structure and one double bond in the side-chain (6β-OH-stigmasterol, 7β-OH-stigmasterol and 7α-OH-stigmasterol). Thus, we can assume that the concentrations of 6α-OH-3-ketostigmasterol, 6β-OH-3-ketostigmasterol, 7-ketostigmasterol, 6β-OH-stigmasterol, 7β-OH-stigmasterol and 7α-OH-stigmasterol, which contain side-chain double bonds, have been overestimated. Quantification of oxides of other common plant sterols, such as sitosterol or campesterol, would not suffer from inaccuracy because they contain the same number of double bonds as the cholesterol oxides used as external standards.

The CV values of oxide contents of six replicate oxidation experiments with duplicate HPLC injections (n=12) were typically below 20%. This indicates that the oxidation experiments were repeatable and the analytical level remained stable. When oxidation proceeded faster, as at 180°C, the variation between the oxidation experiments was smaller. When oxidation was slower, as in the case of 100°C, slightly higher variation between experiments was observed.

In conclusion, the HPLC-UV-FL method proved to be reliable for monitoring sterol hydroperoxides and secondary oxidation products simultaneously. This method is particularly useful when studying the whole oxidation pattern of simple model samples containing only one sterol.

6.1.2 LC-MS

With the LC-MS method, the primary and secondary oxidation products of plant sterols could be analysed without time-consuming sample pretreatment, because no matrix was present. MS detection allowed identification and quantification of also partially or totally co-eluting analytes.

Recently, LC-MS has become more common for the analysis of cholesterol oxides (Sevian et al., 1994; Careri et al., 1998; Manini et al., 1998; Razzazi-Fazeli et al., 2000; Burkard et al., 2004; Raith et al., 2005; Mazalli et al., 2006). Especially LC-MS combined with the soft

ionization technique APCI has become more popular for analysis of cholesterol oxides in biological matrices. Plant sterol oxides other than epoxides have not been analysed earlier by LC-MS.

Compounds with a conjugated diene structure had the protonated molecule ion $[M+H]^+$ as the base peak, which corresponds with earlier results obtained for 7-ketocholesterol (Manini et al., 1998; Razzazi-Fazeli et al., 2000; Raith et al., 2005; Mazalli et al., 2006). The protonated molecular ion was also detectable in the spectra of epoxy and 6-keto compounds. In general, fragments indicating the loss of a variable number of water molecules $[M-H_2O+H]^+$, $[M-2H_2O+H]^+$, $[M-3H_2O+H]^+$ were observed similarly as reported before (Manini et al., 1998; Razzazi-Fazeli et al., 2000; Raith et al., 2005; Mazalli et al., 2006). In the case of 7α-OOH- and 7β-OOH-stigmasterol, fragments representing the loss of hydrogen peroxide $[M-H_2O_2+H]^+$ or hydrogen peroxide and water $[M-H_2O_2-H_2O+H]^+$ were observed, which corresponds to the results of Kusaka et al. (1996) and Kusaka (1993), who reported that hydroperoxides of triacylglycerol had fragments representing loss of water or loss of hydrogen peroxide. The fragmentation behaviour of sterol epoxides differed from fragmentation obtained by Giuffrida et al. (2004) due to the different ionization technique used. They used ammonium formate to enhance the ionization efficiency in ESI. Therefore, they observed an intense molecular ion with an ammonium adduct ion $[M+NH_4]^+$ together with $[M+H]^+$, $[M-H_2O+H]^+$ and $[M-2H_2O+H]^+$.

When the results on fragmentation of cholesterol oxides were compared with those obtained by Manini et al. (1998) and Razzazi-Fazeli et al. (2000), the fragmentation behaviour of the oxides was similar and only relative ion abundances were slightly different. Similar results were also obtained when the fragmentation behaviour of sitosterol, campesterol and stigmasterol oxides was compared with the corresponding oxides of cholesterol; only relative ion abundances differed slightly. Because 6β-OH-3-keto, 6α-OH-3-keto and 7-keto and 5α,6α-epoxy, 5β,6β-epoxy, 6-keto, 6β-OH, 7β-OH and 7α-OH oxides of the same parent sterol had the same molecular weight and especially epimers had similar MS fragmentation patterns, separation between the oxides with different functionalites was essential, as also reported before by Razzazi-Fazeli et al. (2000). Therefore, the identification of sterol oxides with MS requires good separation of oxides with different functionalities. However, the coelution of sitosterol, campesterol, stigmasterol and brassicasterol oxides with the same functional groups was acceptable because they differ in their molecular weights.

The quantification method based on the extracted ion chromatogram of the sum of the main fragments took into account possible small differences present between cholesterol oxides used as standards and the plant sterol oxides analysed. When the linearity of the standard responses was studied, the slope for 7-ketocholesterol was observed to be greater than those for 5α,6α-epoxycholesterol and 7β-OH-cholesterol. Thus, we can assume that the APCI parameters used were slightly more optimal for 7-ketocholesterol than for the other standards. The dry gas and APCI temperature could not be changed during the run. Therefore, the selected temperature of 300°C, optimum temperature for 5α,6α-epoxycholesterol and 7-ketocholestrol, was used. Manini et al. (1998) and Razzazi-Fazeli et al. (2000) also reported that APCI parameters influence signal intensities of cholesterol oxides. The detection limits obtained for cholesterol oxide standards ranged from 0.09 to 0.3 ng/injection, which is similar to the results obtained by Manini et al. (1998) and Razzazi-Fazeli et al. (2000) for cholesterol oxides using LC-MS with an APCI interface. When these detection limits were compared with the amounts of plant sterol oxides in foods, LC-MS can be assumed to be suited to food samples (Table 2). Until now, only GC-FID and GC-MS have been used for analysis of plant sterol oxides in foods.

The LC-MS proved to be a powerful method for the analysis of primary and secondary oxidation products of one sterol or a mixture of sterols. The main advantage of the method was the possibility to identify and quantify partially and totally coeluting analytes. The good chromatographic separation of the oxides of each parent sterol coupled with the specificity of the MS detection enabled analysis of as many as 34 major oxides in a plant sterol mixture.

6.2 Comparison of methods

The HPLC-UV-FL and LC-MS methods were shown to be useful for studying the oxidation pathways of sterols. They enabled monitoring both hydroperoxides and secondary oxidation products. However, with the HPLC-UV-FL method, oxides of only one plant sterol could be analysed. The HPLC-UV-FL method is thus suitable for simple model samples. With LC-MS, the simultaneous analysis of oxides of many sterols is possible. However, both methods require separation of oxides with different functionalities. The quantification of plant sterol oxides is challenging because plant sterol oxides are not commercially available, and thus, the secondary oxidation products of cholesterol were used as external standards for quantification of plant sterol secondary oxidation products, and stigmasterol hydroperoxides were quantified with MeLo-OOH. In the case of the UV detector, the number of double bonds influences the UV absorbance. Because the cholesterol and stigmasterol oxides had a different number of double bonds, the results obtained were not accurate; the method was nevertheless suitable for

studying the oxidation phenomenon. With LC-MS, the quantification was more exact because cholesterol oxides, which were used as standards, were shown to have a similar fragmentation behaviour as plant sterol oxides. The quantification method based on the extracted ion chromatogram of the sum of the three main fragments present also takes into account possible minor differences present in fragmentation of cholesterol and plant sterol oxides. The detection limits in LC-MS analysis were much lower than in HPLC-UV-FL analysis. The CV values obtained with HPLC-UV-FL and LC-MS were similar, but it should be borne in mind that CV values were influenced by both the nature of the oxidation process and the analysis method used.

One important benefit of the LC methods is direct analysis of thermolabile hydroperoxides. Moreover, with LC, operated at room temperature, the risk of artefact formation is decreased. In addition, compared with GC methods, LC methods need less sample pretreatment because non-oxidized and oxidized sterols are better separated. With LC methods, the time-consuming TMS-derivatization step can be avoided. When the LC-MS method was compared with GC-FID and GC-MS methods in quantification of secondary oxidation products of plant sterols in complicated mixtures, all methods gave a similar pattern of oxides during heating.

6.3 Oxidation behaviour of plant sterols

The photo-oxidation and thermo-oxidation experiments were mainly carried out to evaluate the suitability of the LC methods for studying the oxidation mechanism of plant sterols. However, these experiments also gave new interesting results on the formation and decomposition of both hydroperoxides and secondary oxidation products.

6.3.1 Photo-oxidation

This photo-oxidation experiment showed that stigmasterol has a similar photo-oxidation behaviour as cholesterol, consistent with the results of Bortolomeazzi et al. (1999). 5α-OOH-stigmasterol was the main product, followed by 6α-OOH-, and 6β-OOH- and 7α-OOH-stigmasterol. Similar results have been reported for cholesterol (Kulig and Smith, 1973; Smith, 1987; 1996). The formation of 7α-OOH-stigmasterol, a rearrangement product of 5α-OOH-stigmasterol, was observed already at the beginning of the experiment. This rearrangement reaction has been reported to occur during photo-oxidation of cholesterol (Geiger et al., 1997; Yoshida et al., 2003). However, formation of the epimerization product of 7α-OOH-stigmasterol, 7β-OOH stigmasterol, was not observed. When the formation patterns of hydroperoxides during photo-oxidation were studied, the amount of

hydroperoxides was noted to increase during the time. At first, 5α-OOH-stigmasterol was produced at a high rate, but after 30 min content seemed to decrease. This may be due to the rearrangement of 5α-OOH-stigmasterol to 7α-OOH-stigmasterol. Secondary oxidation products were not observed because oxidation had not proceeded sufficiently far.

6.3.2 Thermo-oxidation

When thermo-oxidation of plant sterol was investigated at 100°C and 180°C, hydroperoxides formed with free radical reaction at allylic position C-7 and tertiary position C-25 dominated, as reported earlier for cholesterol (Smith, 1987; Blekas and Boskou, 1999; Lercker and Rodriguez-Estrada, 2002). Distribution of the predominant hydroperoxide was observed to be temperature dependent. At 180°C, formation of 7α-OOH- and 7β-OOH-stigmasterol was more favourable than formation of 25-OOH-stigmasterol. The situation was reversed at 100°C. At 180°C, the formation of hydroperoxides increased sharply during the first 10 min and then started to decrease due to decomposition of hydroperoxides to secondary oxidation products. After 10 min, the amount of all secondary oxidation products increased sharply. Most of the secondary oxidation products reached a plateau or even decreased after 30-40 min of oxidation. By contrast, at 100°C, the amounts of both hydroperoxides and secondary oxidation products increased over the entire seven-day oxidation period. The increase of hydroperoxides become slower after three days of oxidation because hydroperoxides partly decomposed to secondary oxidation products. As noticed in this study the heating time and temperature have an effect on the formation rate and formation patterns of plant sterol oxides; the same products were formed at both temperatures, but their distribution differed.

The main secondary oxidation product formed was 7-ketosterol, followed by 5β,6β-epoxy-, 7β-OH, 5α,6α-epoxy-, 7α-OH- and 6β-OH-3-keto-sterols, the order of which depended on the temperature or analysis method used. An explanation for the high amount of 7-keto compounds formed and the oxidation profile change during the oxidation process could be that 7-OH compounds of sterols can be dehydrogenated to 7-keto compound under dry and oxygen-rich conditions, as reported to happen with 7-OH-cholesterol (Teng et al., 1973; Chien et al., 1998). The formation of 7β-OH-sterol seems to be more favourable than that of 7α-OH-sterol at all temperatures studied. This can result from the lability of 7β-OOH-sterol toward thermal decomposition to 7β-OH-sterol (Teng et al., 1973). Paniangvait et al. (1995) observed that 7β-OH-cholesterol predominanted over 7α-OH-cholesterol due to the greater thermodynamic stability of 7β-OH-cholesterol. Giuffrida et al. (2004) reported that sterol epoxides are formed by a bimolecular reaction mechanism by interaction of a peroxyl radical and an unoxidized sterol. Because epoxides were observed at the first time-point, sufficient

peroxides can be assumed to be present for epoxy formation during the entire heating period. The 5β,6β-epoxysterol was dominant compared with the 5α,6α-epoxysterol. This may be due to the steric hindrance of the OH group at position C-3 (Smith, 1987). Minor independent reactions that can occur during autoxidation are formation of 6β-OOH-3-keto and 6α-OOH-3-ketosterols (Smith, 1987). At the temperatures and oxidation conditions of this study, this reaction mechanism seemed to be less favourable. No triol compounds were observed due to the lack of water in the system (Zhang et al., 2005)

The results showed that all plant sterols with one double bond in the ring structure thermo-oxidize similarly, because the proportion of each plant sterol in the oxides quantified was similar to that in the unoxidized mixture. Therefore, we could assume that side-chain double bonds of stigmasterol and brassicasterol did not change their reactivity or the difference between the reactivities was too low to be noticed.

In this research, the focus was on the determination of hydroperoxides and secondary oxidation products of sterols, which are more polar than unoxidized sterol. These products have been suggested not to account for all losses of plant sterols (e.g. Kim and Nawar, 1993; Soupas et al., 2005). Especially at high temperatures, other products such as non-polar compounds, dimers and polymers are also formed. This study, however, focused on the critical products, which are formed during the early stages of oxidation and are subjected to further potentially harmful reactions. Elucidating these products is essential for preventing and controlling oxidation.

7. CONCLUSIONS

The HPLC-UV-FL and LC-MS methods were developed for investigation of oxidation reactions of plant sterols. The applicability of the methods was examined by using photo- and thermo-oxidized stigmasterol or thermo-oxidized sterol mixtures as model samples.

NP chromatography was used in both LC methods because of its ability to separate positional and epimeric isomers of plant sterol oxides. With both methods, separation of oxides with different functional groups is required. Depending on the polarity range of oxidation products present, isocratic or gradient elution is needed. In photo-oxidation, oxides formed can be separated with isocratic elution, whereas in thermo-oxidation gradient elution is needed to separate oxides with different polarities.

The HPLC-UV-FL method allows the investigation of both primary and secondary oxidation products of plant sterols. Hydroperoxides are detected specifically by the FL detector after post-column reagent addition, and the UV detector can generally detect all oxides. Specific detection and quantification of hydroperoxides is the greatest advantage of this method, enabling early reactions of oxidation to be investigated. The HPLC-UV-FL method is particularly suitable for studying the reactions of one sterol at one time and is thus useful for simple models. The requirement of separating all analytes restricts its use in complex sterol oxide mixtures. Because no plant sterol oxides are commercially available, compromises need to be made in quantification. The number and position of double bonds should be the same in the standard and the analyte to obtain accurate quantification using UV detection.

The major advantage of MS detection coupled with LC is the capability of identifying and quantifying analytes even in the case of incomplete separation. Simultaneous analysis of oxides of several plant sterols is therefore possible. However, oxides with the same molecular mass have to be completely separated. Here, fragments indicating a loss of different numbers of water molecules were obtained. The protonated molecule ion was the base peak only in the case of conjugated dienes. Results revealed that all plant sterols and cholesterol have similar fragmentation behaviour; only relative ion abundances are slightly different. Therefore, quantification can be done using cholesterol oxides as external standards. Furthermore, the quantification method used, which was based on the extracted ion chromatogram of the sum of the main fragments, took into account potential minor differences between cholesterol oxides and plant sterol oxides.

Both the HPLC-UV-FL and LC-MS methods were shown to be useful for studying the oxidation reactions of plant sterols, enabling the following of the formation and decomposition of individual hydroperoxides and secondary oxidation products. The HPLC-UV-FL method showed different formation rates of hydroperoxides during photo-oxidation. Furthermore, the methods revealed that stigmasterol has a similar photo-oxidation behaviour to cholesterol, and all plant sterols investigated had similar thermo-oxidation behaviours. In addition, the predominant reactions and oxidation rates in thermo-oxidation were revealed to be temperature dependent.

In conclusion, the results presented here demonstrate that the HPLC-UV-FL and LC-MS methods can be used to investigate the oxidation mechanism of sterols. Future research should focus on studying the oxidation mechanism of plant sterols in more detail. In addition, developing the methods for more complex models, including different matrices, is essential. These findings can serve as a useful foundation for designing methods for more complicated systems.

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